ISOLATION AND IDENTIFICATION OF MALAYSIAN *PASTEURELLA* SPECIES RESPONSIBLE FOR SMALL RUMINANT PNEUMONIA FOR THE PURPOSE OF DEVELOPING AN EFFECTIVE STRAIN-SPECIFIC VACCINE

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CONTENTS

DECLARATION

The work reported in this thesis was part of a larger project concerned with investigations of pasteurellosis in sheep, and consequently some of the experimental findings were obtained in collaboration with my colleagues at the Moredun Research Institute. Nevertheless, most of the work presented in this thesis was carried out by myself, and where conjoint experiments were necessary, a full role was played in the design of the experiments and the interpretations of the results.

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ABSTRACT OF THESIS

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Title of Thesis Isolation and identification of Malaysian *Pasteurella* species responsible for small ruminant pneumonia for the purpose of developing an effective strain-specific vaccine

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The aims of this study were to identify the prevalance and distribution of *Pasteurella haemolytica* serotypes causing pneumonic pasteurellosis in sheep and goats in Malaysia, assess the efficacy of a novel *P. haemolytica* vaccine in field trials and to isolate, characterise and assess the immunological significance of the polysaccharide capsule of *P. haemolytica* A2. The serotyping study indicated that there was little difference in the relative frequency of occurence of A serotypes in the UK and in Malaysia. Serotype A2 was by far the most common and the other A serotypes did not differ significantly in order of prevalence. However, T biotypes appeared to be very rare in Malaysia compared to UK. When electrophoretic protein and lipopopolysaccharide profiles of some common strains from both countries were compared there appeared to be no significant differences among the strains. Four to five major protein bands with about twenty minor bands were shown to be present. The lipopolysaccharides profiles were of rough-type.

A new *P. haemolytica* iron-regulated protein (IRP) vaccine was tested in field studies for its efficacy in preventing pasteurellosis in Malaysian sheep farms. The results showed that this vaccine generated an immune response as measured in ELISA and IHA tests. The antibody titers were significantly higher in the vaccinated sheep and there appeared to be protection against clinical pasteurellosis following vaccination.

The capsular polysaccharide antigen of *P. haemolytica* A2 was identified as an important immunogen and as a candidate for a future *Pasteurella* vaccine. An outer membrane protein-polysaccharide (OMP-PS) complex was succesfully prepared from an ovine isolate of *P. haemolytica* serotype A2 by precipitation from log phase culture supernatant and subsequent purification by column chromatography. The optimum production of the complex was determined to be in 6 hour culture and comprised protein and polysaccharide (4:1 w/w) with low in lipopolysaccharide content.

This complex was immunogenic in mice and adult sheep with induction of humoral anti capsular antibody in indirect heamagglutination (IHA) test and anti-OMP antibodies in immunoblotting. Direct immunisation of mice with the complex vaccine demonstrated significant protection against A2 infection. The sera from two-year old sheep immunised with OMP-PS passively protected mice against A2 challenge. Three-month old lambs immunised with the same vaccine did not respond serologically and this sera did not passively protect mice.

In vitro studies using ovine and murine macrophages indicated that the mechanism of protection is antibody mediated phagocytosis as the opsonophagocytic activity of immune sera could be demonstrated. These results indicate the potential of a *P. haemolytica* A2 OMP-PS complex vaccine to immunise adult sheep and suggests that passive protection of lambs against A2 infection is obtainable.

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I am particularly indebted to to Mr. Steeve and his staff for provision of glassware and media Mr. B. Easter, Mr. A. Inglis and Dr. D. Buxton for preparation of photographs, Mr. Mike, Mrs. Audrey, Dr. Basiro and Dr. Mahmud for advice on statistics and computing. Many thanks to the staff of small animal units for providing and looking after experimental animals. I am grateful to all members of staff of MRI who were involved in any way with this project. To 'tablighi' brothers, your continuous 'zehin' was appreciated.

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ABBREAVIATIONS USED IN TEXT

BAM	- Bronchoalveolar macrophage
BHI	- Brain heart infusion
BWB	- Blot wash buffer
DAB	- Diaminobenzidine
DMSO	- Dimethyl sulphoxide
DVS	- Department of Veterinary Services
ELISA	- Enzyme-linked immunosorbent assay
FITC	- Fluorescein thiocynate
FPBS	- Formolised phosphate buffered saline
HRP	- Horse-radish peroxidase
IHA	- Indirect haemagglutination
IRP	- Iron-regulated proteins
Ig	- Immunoglobulin
LPS	- Lipopolysaccharide
mAb	- Monoclonal antibodies
MALIN	- Malaysian indigenous sheep
MARDI	- Malaysian Agriculture Research and Development Institute
MRI	- Moredun Research Institute
NB	- Nutrient broth
ND	- Not done
OD	- Optical density
OMP	- Outer membrane protein
PAGE	- Polyacrylamide gel electrophoresis
PBS	- Phosphate buffer saline
PBSS	- Phosphate buffer saline solution
PI3	- Parainfluenza virus type 3
PS	- Polysaccharide capsule
RBC	- Red blood cell

SAPU	- Scottish Antibody Production Unit
SDS	- Sodium dodecyl sulphate
SE	- Standard error of means
SPF	- Specific pathogen free
SSE	- Sodium salicylate extract
TSI	- Trypticase soya broth
TRIS	- Tris buffered saline
UV	- Ultra violet

CHAPTER 1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

Pneumonic pasteurellosis in sheep is an important disease and causes serious economic losses to the livestock industry throughout the world. In the United Kingdom a survey conducted by the Ministry of Agriculture, Fisheries and Food (MAFF, 1964) showed that 82% of all carcasses referred to Veterinary Investigation Centres showed evidence of pneumonia. The veterinary investigation report for Scotland for the years 1976-1977 showed that most sheep pneumonia is related to pasteurellosis (Veterinary Investigation Diagnosis Analysis Report, 1977). The organism most frequently isolated from the pneumonic lungs is *P. haemolytica* (Gilmour, 1978a). In South Africa, Van der Veen and Zumpt (1976) recovered *P. haemolytica* from 13 out of 36 outbreaks of pneumonia and *P. multocida* from six. Dennis (1974) indicated that *P. haemolytica* was the main cause of perinatal lamb mortality in Western Australia. *P. haemolytica* was also most commonly isolated from healthy flocks in United States of America (Frank, 1982) and pneumonic lungs in New Zealand (Prince *et al.*, 1985).

Pneumonia has also been associated with other types of bacteria such as *Escherichia coli*, *Staphylococcus* spp., *Streptococci* spp., *Pseudomonas* spp. and *Fusobacterium necrophorum*, but they are thought to be of minor importance.

Pneumonic pasteurellosis is not restricted to sheep and goats, but can infect a wide range of animals such as cattle, swine, horses and fowl. Since the early 1900s bovine pneumonic pasteurellosis has been recognised as a major economic problem to European and North American cattle industries (Yates, 1982). McKercher (1978) estimated losses in the United States due to the disease also known as "shipping fever" at 76 million dollars in 1972 and in 1981 the annual losses amounted to approximately 800 millions dollars (Drummond *et al.*, 1981). The involvement of *P. haemolytica* in the cattle pneumonia has been well documented (Carter, 1967; Gilmour, 1978).

While some efforts have been directed towards minimising exposures to certain factors commonly associated with the disease, vaccination is accepted as the most effective and practical method of control. Numerous studies undertaken over the past decades have evaluated the efficacy of *Pasteurella* vaccines composed of whole organisms and or various sub-cellular preparations. In spite of these efforts, vaccines have had dubious beneficial effects and pneumonic pasteurellosis remains one of the most significant causes of loss in sheep population of the United Kingdom (Gilmour, 1978) and Malaysia (Mohamad, unpublished data, 1993).

Gilmour *et al.* (1983) showed that protection with the available commercial vaccines is variable with little or no protection being provided by the vaccines containing sodium salicylate extracts (SSE) against heterologous serotype challenge. This indicates that either the immunising power of the commercial vaccines is insufficient to protect against the disease or the vaccines might not contain the appropriate range of serotypes and that the antigens might not be incorporated in the optimum way.

A number of antigens have been assessed as potential immunogens. Gilmour *et al.* (1979) has shown that vaccines prepared from extracts of bacterial cells do protect sheep against experimentally produced pneumonic pasteurellosis. The homologous vaccines incorporating sodium salicylate extracts (SSEs) have protected sheep against disease caused by biotype A serotypes 1, 6 and 9. However, vaccines containing SSE, or heat-killed cells of serotype A2 (the serotype of most importance and which is responsible for the majority of the outbreaks of ovine pasteurellosis) have been less effective immunogens in mice, rabbits and sheep (Gilmour *et al.*, 1983; Evans *et al.*, 1979b; Donachie *et al.*, 1986). One reason for this may be the poor immune response to the serotype-specific capsular polysaccharides which are a common constituent of host membranes (Adlam *et al.*, 1987). A more detailed knowledge of the important antigens involved in stimulating protection could lead to improvement in the production and the efficiency of existing commercial vaccines. Recent work by many investigators has determined that a number of antigens are potential immunogens; these include:

i) Capsule

ii) Outer Membrane Protein (OMP)

iii) Lipopolysaccharide (LPS)

iv) Leukotoxin (cytotoxin)

v) Iron-Regulated Protein (IRP)

vi) Fimbrae

vii) Neuraminidase

viii) Haemolysin

ix) Protease

1.2 Pasteurellosis in sheep and goats with reference to Malaysia.

Malaysia is a tropical country with a total land area of 329 758 km^2. She has two main weather seasons, the dry season from Mac to August and the rainy seasons from October to Febuary. The temperature varies between 23°C and 33°C with an average of about 26°C.

In 1993, the estimated ruminant population was 689,288 cattle, 110,149 buffallo, 244,023 sheep and 277,065 goats (DVS Report, 1993). Extensive or free-range grazing systems in rural areas, and semi-intensive and intensive systems in organised farms are used in cattle, sheep and goat husbandry. For sheep and goats, integration with plantation crops such as rubber and oil pallm is expanding. The animals are allowed

to graze native grasses under the tree crops for about 7 hours. They are housed at night where water and supplementary feed are provided.

The main causes of sheep and goat mortality in Malaysia are pneumonic pasteurellosis, haemonchosis, pregnancy toxaemia and blue tongue. Pneumonic pasteurellosis is by far the most important and accounts for more than 35 percent of sheep mortality (Wan Mohamad *et al.*, 1988). Another report estimates that the pneumonia-bluetongue complex causes more than 50 percent of deaths in newly imported sheep in Malaysia (Hadi, 1988). Evidence provided by various investigators indicates that two species are the main causative organisms of pasteurellosis. These are *Pasteurella haemolytica* and *Pasteurella multocida,* with the former being by far the more significant (Gilmour, 1978a).

Pasteurellosis affects sheep and goats of all ages under all management conditions. *Pasteurella* species are normal flora in the upper respiratory tract and tonsils of most sheep and goats. Management practices such as castration, docking or infections with para-influenza virus, mycoplasma, mould or the agent of tick-borne fever are among the predisposing factors thought necessary to precipitate pasteurellosis (Gilmour *et al.*, 1980). The hot humid tropical climate of Malaysia, with unpredictable rainy spells and monsoons, aggravates the condition to the disease. Depending on these predisposing factor(s) and the strains of the organisms involved the disease can be acute or hyperacute as well as chronic (Gilmour *et al.*, 1980).

In Malaysia, pneumonic pasteurellosis may be regarded as a clinical manifestation of an infection which occurs as the result of various stress factors such as transportation of sheep and goats from place to place. There have been no reported attempts to determine the incidence of the disease which occurs under the type of management systems used in Malaysian sheep farms. The disease occurs frequently in young lambs and kids with the highest incidence during or shortly after the rainy season (personal observation). In severe outbreaks of pasteurellosis, more than 50% of the sheep in a flock may be affected and death rates are usually more than 25% (Mohamad, 1993; unpublished data).

Sheep and goat pasteurellosis in Malaysia is usually caused by *P. haemolytica* and occasionally by *P. multocida* (Sheikh Omar *et al.*, 1989). The occurence and severity of the disease may depend on a series of complex interactions between several infectious agents, environmental factors, and the immunological status of the lamb.

Although in Malaysia antimicrobial agents have been extensively used to treat pneumonic pasteurellosis, vaccination has been accepted and is becoming more popular. The types of vaccines used include formalin-killed whole cell vaccines, extracts of whole cells or live attenuated vaccines. Several kinds of commercially prepared broth vaccines have been used in Malaysia, but their efficacy in the field has been questioned (Wan Mohamad *et al.* , 1988).

Some of the commercial vaccines used widely in Malaysia are Heptavac^{R} (Hoechst, UK) and Carovac^{R} (Pitman-Moore, UK). Despite the widespread use of the vaccines, outbreaks of pasteurellosis still happen and there are no indications that the prevalence of the disease is diminishing (Gilmour, 1980). Indeed, vaccinated animals are frequently more apparently susceptible to the disease than their non-vaccinated counterparts (Shewen & Wilkie, 1982).

1.3 Objectives of the Study

This thesis concentrates on *P. haemolytica* serotype A2 as the main cause of ovine and caprine pneumonic pasteurellosis. The first part reviews the literature associated with *P. haemolytica*, the disease and prevalence and distribution of serotypes in Malaysia. The second part is concerned with the testing of a new *P. haemolytica* IRP vaccine (Ovipast-9-IRP^{R}, Hoechst, UK) for its efficacy on Malaysian sheep farms. The final part deals with the isolation, characterisation, purification and assesment of the immunological significance of the polysaccharide capsule of *P. haemolytica* A2.

CHAPTER 2.0 LITERATURE REVIEW

2.1 The characterisation of *P. haemolytica*

2.1.1 The Bacterium

The first description of the organism was that of Jones (1921) as a short, encapsulated Gram-negative, non-motile cocco-bacilli (Plate2.1). Colonies on blood agar are flat and translucent, 3-5 mm in diameter after 48 h incubation and show a weak haemolysis on 7% sheep blood agar (Plate 2.2). The organism is aerobic and facultatively anaerobic. It is oxidase and catalase positive and attacks sugars by fermentation yielding small amounts of acids without producing gas (Cowan & Steel, 1974). It can be distinguished from other members of the genus *Pasteurellla* using the characteristics shown in Table 2.1.

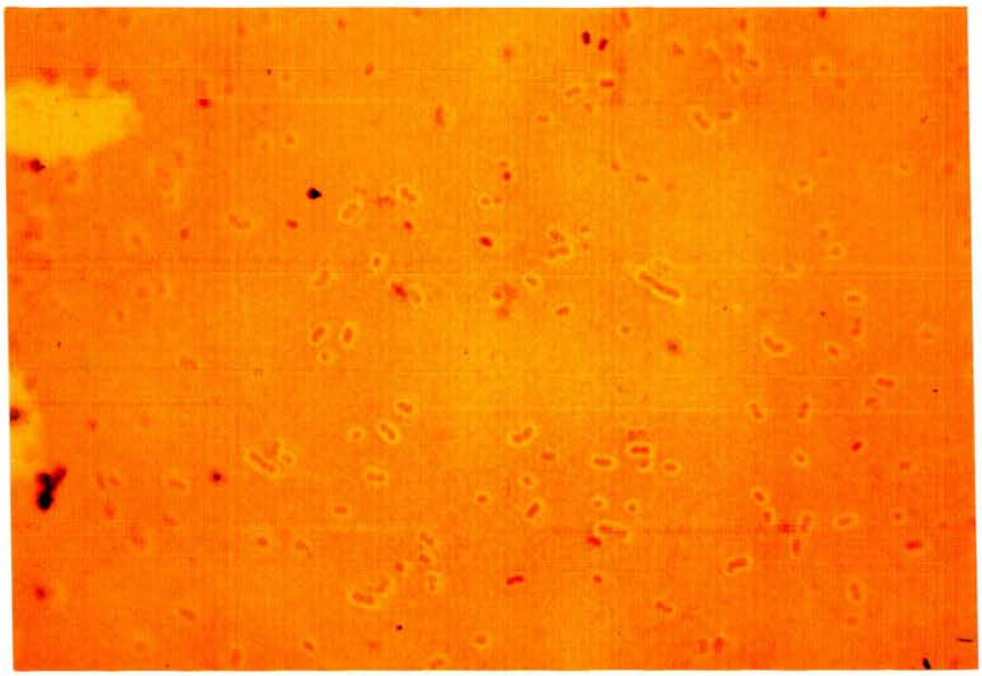

Plate 2.1. **Colonies of *P. haemolytica* A2; encapsulated, the most predominant field isolates in pneumonic pasteurellosis.**

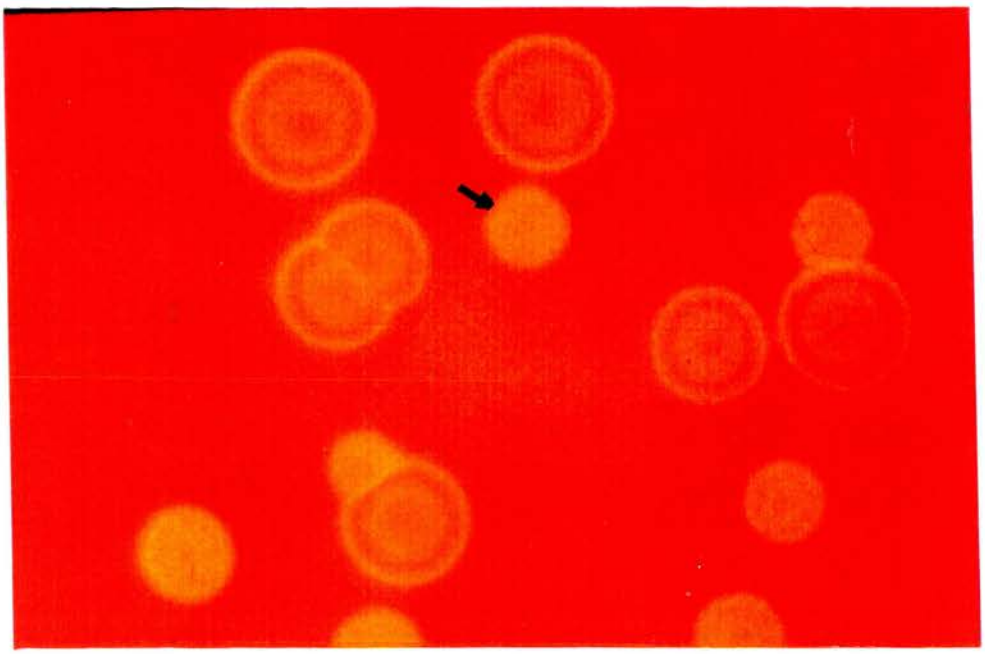

Plate 2.2. **Colonies of *P.haemolytica* A2 on blood agar plate (small colonies are arrowed; the larger colonies are T strains). The cell measures about 0.7 μm in diameter.**

Table 2.1. Differentiation of *P. haemolytica* from other *Pasteurella* species.

1.	*P. haemolytica* causes a narrow zone of haemolysis on 7% ovine or bovine blood agar. Other *Pasteurella* species are not haemolytic.
2.	*P. haemolytica* grows on MacConkey agar whereas other *Pasteurella* species will not.
3.	*P. haemolytica* is unable to produce urease and indole where as other *Pasteurella* species do.

2.1.2 Biotyping

Strains of *P. haemolytica* can be classified into two biotypes, A and T, differentiated on the basis of several *in-vitro* biochemical criteria including carbohydrate fermentation activity (Smith, 1961), nucleic acid homology (Biberstein and Francis, 1968) and antibiotic sensitivity (Biberstein and Kirkham, 1979).

Smith (1961) investigated the biochemical characteristics of *P. haemolytica* and showed that in sugar fermentation two distinct biotypes (A and T) of *P. haemolytica* can be differentiated. Biotype A isolates mainly ferment arabinose and biotype T isolates mainly ferment trehalose. These two biotypes are responsible for

different clinical forms of pasteurellosis in sheep, goats and cattle. The A biotypes are associated with pneumonic pasteurellosis, whereas the T biotypes have been associated with systemic disease in young lambs (Smith, 1961).

The ability of strains to ferment xylose, salicin and lactose also assist in differentiating between biotypes. Biberstein (1978) showed that A biotypes are usually salicin negative, xylose positive and lactose positive (apart from serotype A2 strains which are usually salicin positive and negative for xylose and lactose).

In terms of antibiotic susceptibility, Smith (1961) showed that biotype A isolates are generally more susceptible to penicillin than those of biotype T. This has been confirmed by Biberstein and Kirkham (1979) who showed that biotype A serotypes also had an increased *in vitro* susceptibility to ampicillin, cephalotin, chloramphenicol, tetracyclin, erythromycin and nitrofurantion. A strains are also more susceptible to growth inhibitors such as fuchsin, methylene blue and brilliant green (Olmos and Biberstein, 1979).

The biotype A isolates may be differentiated from biotype T isolates by the colony morphology on blood agar. At 24 h, biotype A isolates appear as evenly coloured grey colonies, whereas more of biotype T produce larger colonies with large brownish centres (Adlam, 1989 and Plate 2.2). In DNA-RNA hybridization experiments, Biberstein and Francis (1968) have shown that there was a low degree of relationship

between representatives of A and T strains, but a high relationship between the two A strains tested. Davies (1994) have recently shown that the biotype A may be differentiated from the biotype T from the analysis of their OMP and LPS profiles using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting techniques.

The genome of *Pasteurella* species has also been studied in attemps to establish relationships between strains. More recently, nucleotide sequencing has proved to be a superior method of genome analysis and strain differentiation. The cloned genes could be used as specific probes in hybridization studies into the organization and distribution of the virulence antigens in the bacteria. Lo, (1994) showed that the A and T biotypes carry a very different genetic organization for these genes.

2.1.3 Serotyping

Biberstein *et al.* (1960) first developed an indirect haemagglutination assay (IHA) to subdivide biotypes A and T strains into different serotypes. This method of serotyping relies on the differences in capsular polysaccharides present on the strains and to date 17 serotypes have been identified with thirteen of these belonging to the A biotype: A1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14 and 16 and four belonging to the T biotype T3, 4, 10 and 15 (Fodor *et al.*, 1987; 1988; Younan *et al.*, 1995). Adlam *et al.*, (1984, 1985a, 1985b, 1986, 1987) have purified and characterised five of the serotype specific

capsular polysaccharides.

A number of isolates cannot be serotyped using these methods. Fraser *et al.* (1982) reported that 6-11% of ovine isolates were untypable. These strains have been isolated from both healthy and diseased sheep. Quirie *et al.* (1986) noted that the percentage of untypable strains was higher in cattle and that most isolates were associated with the nasopharynx of healthy animals or with the female genital tract (including the placenta and foetus), udder and milk. Aarsleff *et al.* (1970) showed that these untypable strains generally belong to the A biotype and that their negative reaction in the IHA test is due to lack of the soluble antigen responsible for serotyping. According to Biberstein (1978), untypable strains probably represent unencapsulated mutants and probably belong to the A biotype since they exhibit sugar fermentation patterns more typical of A strains than of T strains.

Serotyping of *P. haemolytica* strains can also be carried out by different techniques, which include a rapid plate agglutination method developed by Frank and Wessman (1978) where direct agglutination of the bacterial cells can be carried out on a microscope slide. The method suggests that the serotyping antigen is a polysaccharide and present on the surface of the bacterium. Chengappa *et al.* (1984) described counter-immune electrophoresis (CIE) method which involves two dimensional electrophoresis that seperates antigens by their charge in the first dimension then visualises them by precipitation against specific antiserum in the second dimension. Other methods include

a modification of IHA techniques by the use of microtitre trays (Shreeve *et al.* 1972) and glutaraldehyde fixed-ox cells (Fraser *et al.*, 1982, 1983).

2.2 *P. haemolytica* Antigens

Structurally *P. haemolytica* resembles other Gram-negative organisms in having several cell wall associated components or products which help it to become established during an infection (Figure 2.1). In addition to serotype specific capsular polysaccharides it possess lipopolysaccharides, outer and inner membrane proteins and peptidoglycan (Adlam, 1989). A glycocalyx and fimbrae have been reported recently. Two types of fimbrae or pili, one thick and rigid and the other thin and flexible are present on the surface of A1 serotype organisms grown in agar (Morck *et al.*, 1987; Potter *et al.*, 1987). Apart from cell wall associated antigens, the organism produces enzymes and proteins which include a potent and specific cytotoxin, a haemolysin, and extracellular enzymes including neuraminidases and proteases. Many aspects of the structure and function of these antigens remain to be elucidated.

The structure and function of the bacterial cell surface antigens, as well as their role in pathogenesis and immunity are subjects of great interest to scientists, as this knowledge is essential for the development of effective *Pasteurella* vaccines. A number of antigens have been assessed as potential immunogens and a number of advances have been made in the formulation of possible future vaccines. The important antigens which

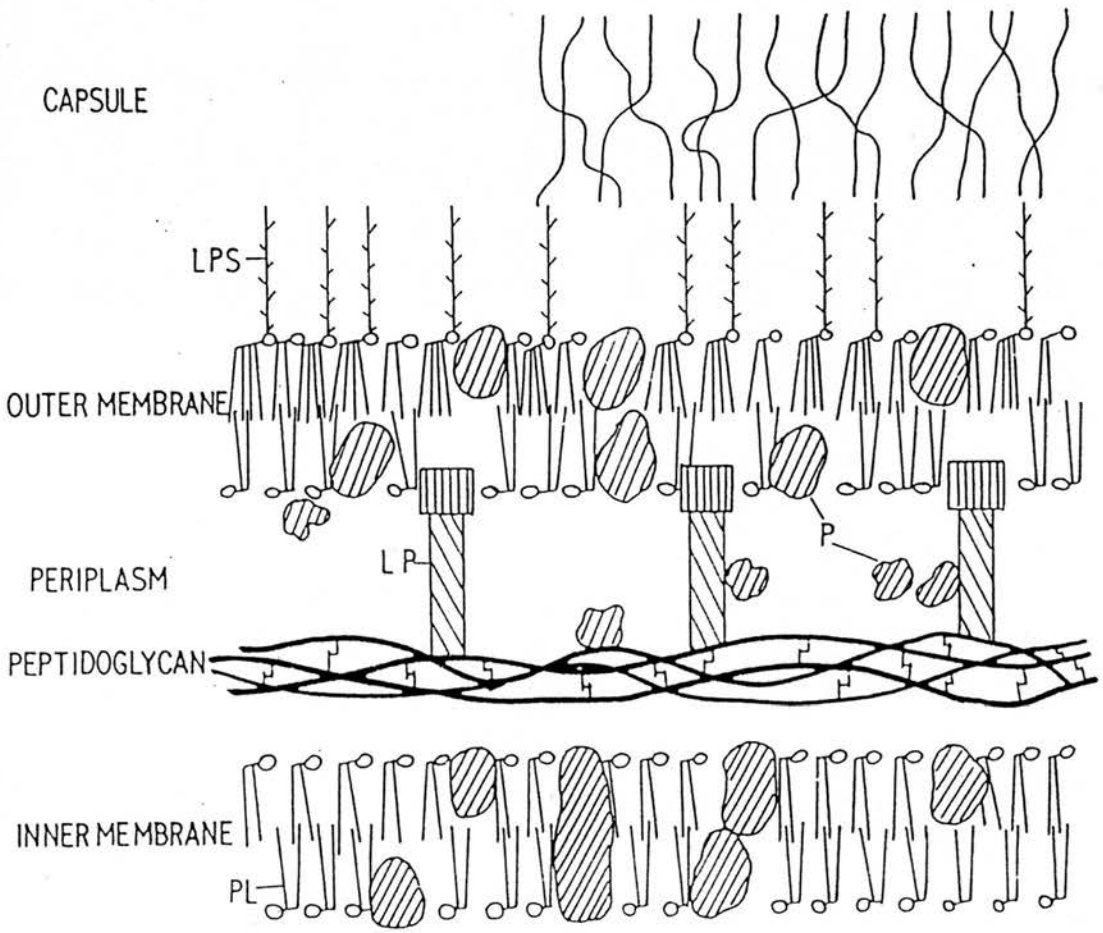

Figure 2.1. Schematic representation of the Gram-negative cell envelope.

LPS - Lipopolysaccharide LP - Lipoprotein

P - Protein PL - Phospholipid

Plate 2.3. **Electron micrograph of the *P. haemolytica* serotype A2, showing the location of capsular materials (Arrowed).**

have been studied are briefly mentioned here, but this thesis will concentrate on the capsule and outer membrane protein of *P. haemolytica* serotype A2 as future components of a *Pasteurella* vaccine.

2.2.1 Capsule

P. haemolytica possesses a soluble, heat stable external capsule composed of extracellular polysaccharide. It is responsible for serological specificity and has long been recognised as playing a role in the pathogenicity of the organism (Biberstein *et al.*, 1960). It is loosely attached to the bacterial cell and can be removed by vigorous shaking or alkali treatment. The capsule is very hydrated, being only 1-2% polysaccharide and probably functions as a moisture and ion trapping layer around cells and may inhibit phagocytosis (Donachie, 1984a). Being negatively charged it can be stained with ruthenium red (Howard & Gourlay, 1974) or labeled with polycationic ferritin and examined by electron microscopy (Weiss *et al.*, 1979; Gilmour *et al.*, 1985) (Plate 2.3).

Exopolysaccharide can be divided into homopolysaccharides and heteropolysaccharides depending on the composition and the complexity of the sugar groups in the polymer. Neutral hexases such as D-glucose, D-galactose and D-mannose are frequently present in the capsule. Sialic acid polymers, a type of homopolysaccharide (N-acetylneuraminic acid or NANA), are found in *P. haemolytica* serotype A2, *Neisseria meningitidis, Escherichia coli* K1 and *Moraxella liquafaciens* (Bovre *et al.*, 1983). It is a colominic acid structure α-(2→8)-linked polymer of N-acetylneuraminic acid or NANA)

(Figure 2.2). In mammals, linear polymers of α-(2→8)-linked NANA form surface glycoproteins of mammalian neural cells (Finne *et al.*, 1983), neural cell adhesion molecules (Finne *et al.*, 1985) and gangliosides (Soderstrom *et al.*, 1984).

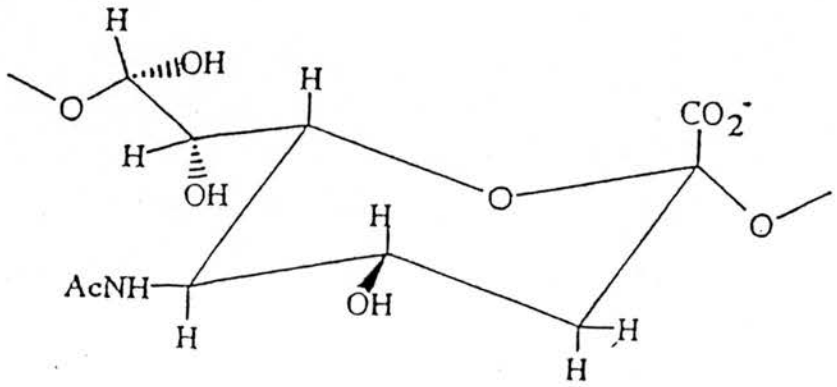

Figure 2.2 Structure of capsular polysaccharide of *P. haemolytica* A2; an α(2→8)-linked N-acetyl neuraminic acid (NANA).

The polymers of NANA confer the property of invasivesness to *E. coli* K1 (Schiffer *et al.*, 1976). In infant rats, *E. coli* K92 strains induce bacteremia and meningitis after intestinal colonisation (Glode *et al.*, 1977). Passive administration of as little as 1μg of H 46 or human immunoglobulin M monoclonal antibodies specific for α-(2→8)-linked NANA protected the infected rat pups from bacteremia and death.

Unfortunately this polymer has been shown to be poorly immunogenic when used as a vaccine component against *N. meningitidis* group B disease in human beings (Lifely *et al.*, 1987) and did not induce an antibody response in lambs (Adlam *et*

al., 1987). The polymer alone, or complexed to homologous outer membrane protein, induced low transient levels of IgM antibody in mice and is relatively short lived. (Moreno *et al.,* 1983). Covalent attachment of this capsular polysaccharide to a heterologous protein failed to elicit antibody to this antigen. Hypotheses have been postulated to explain the poor immunogenicity of this polysaccharide, such as sensitivity to the host neuraminidases, cross-reactivity with self antigens and loose configuration of the purified polysaccharide (Lifely *et al.*, 1987).

Although there exists controversy regarding the protective antigens of group B *N. meningitidis*, there is little doubt as to the importance of its capsular polysaccharides as virulence factor. Lifely *et al.* (1987) have identified the capsule of *N. meningitidis* group B and *E. coli* K1 as a virulence factor and protective antigen. Using monoclonal antibodies with specificities for *N. meningitidis* group B capsule and outer membrane protein-polysaccharide complex meningococcal vaccine, (Donachie 1991, unpublished data) has shown protection against *P. haemolytica* A2 challenge in mice. These results prompted research to formulate a *P. haemolytica* A2 vaccine with a similar composition to the meningococcal vaccine to evaluate its efficacy against experimental pasteurellosis.

2.2.2 Outer Membrane Protein (OMP)

The proteins of the outer membrane are arranged as shown in Figure 2.1. They are embedded in the lipid bilayer and typically make up almost half the dry weight of an outer membrane. Nikaido and Nakae (1979) in analysing the outer membrane protein found that the outer surface of the membrane is 59% protein and 41% LPS while the inner surface composed of 53% phospholipid and 47% protein.

The study of the protein of the outer membrane has been well documented and many reviews are available in the literature (Di Rienzo *et al.*, 1978). Techniques are available to visualise and resolve the protein components of the outer membrane and purification of the proteins has been achieved by selective solubilization with detergents such as Sarkosyl (Filip *et al.*, 1973), by gel permeation chromatography or by electroelution from the polyacrylamide gel following Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Commassie blue or silver staining (Tsai & Frasch, 1982) of outer membrane fractionated on SDS-PAGE reveals the OMP profile of the bacterium, which normally comprises several heavily stained bands (the major OMPs) and numerous minor bands. Knight *et al.* (1990) have shown that envelopes isolated from *P. haemolytica* biotype A, *P. haemolytica* biotype T and *P. multocida* could be differentiated by their SDS-PAGE profiles. The authors further showed that different isolates of the same *P. haemolytica* A serotype always generated very similar SDS-PAGE profiles and *P. haemolytica* T envelopes gave simpler SDS-

PAGE profiles which were typified by distorted bands in the high molecular mass region. Thompson & Mould (1975), who analysed phenol/acetic cell extracts of *P. haemolytica* by SDS-PAGE and showed differences in the profiles of extracts derived from A strains and T strains. *E. coli* outer membrane protein has received most attention and work by Ames (1974) showed that between 36 and 66 protein bands could be identified in its outer membrane of which 4 or 5 appeared to be major components. Investigation into other species such as *Salmonellla typhimurium* and *Neisseria gonorrhoeae* have shown similarities in that 4-5 major proteins are always present (Bragg and Hou, 1972; Heckels, 1977). Squire *et al.* (1984) have separated and purified membranes of A1 serotypes and showed that major OMP had molecular masses of 30 and 42 kDa.

2.2.3 Lipopolysaccharide (LPS)

The biological activities of LPS as a virulence determinant in Gram-negative bacteria have been thoroughly investigated. LPS confers resistance to complement and bactericidal activity of serum. It stimulates the release of mediators involved in the inflammatory response, the coagulation pathway and damage of tissue (Morrison and Leive, 1978). Keiss *et al.* (1964) estimated that between 12 and 15% of dried cell weight of *P. haemolytica* consisted of LPS. A study conducted by Tsai *et al.* (1982) showed that water extracts of *P. haemolytica* when compared by crossed-immunophoresis revealed a distinct serological difference between LPS molecules extracted from different serotype strains.

extracted from different serotype strains.

The LPS of *P. haemolytica* has similar biological activity to the LPS of other Gram-negative bacteria such as *S. typhymurium* and *E. coli*.. It showed a similar endotoxic effect when given to chick embryos and in limulus amoebocytelysate tests (Rimsay *et al.*, 1981). It has been shown that *P. haemolytica* LPS binds to sheep lung surfactant and causes lesions similar to those found in natural disease (Brogden *et al.*, 1986). The bactericidal effect of immune sheep sera to A2 serotype can be removed by absorption with purified LPS, indicating that this is the target antigen and that it is important in immunitiy as well as in pathogenesis (Sutherland, 1988). Sutherland *et al.* (1990) also reported that LPS composition differs between *in vivo* grown bacteria and those grown *in vitro*, and these differences are associated with diffferences in relation to opsonophagocytosis and complement-dependent killing. LPS can also stimulate TNF-α release from bovine alveolar macrophages (Bienhoff *et al.*, 1992).

2.2.4 Leukotoxin (cytotoxin)

The leukotoxin of *P. haemolytica* has specificity for leukocytes of sheep (Sutherland *et al.*, 1983), cattle (Benson *et al.*, 1978) and goats (Chang *et al.*, 1987) and has been recognised as a major pathogenic determinant of *P. haemolytica*. It has been shown to be sensitive to heat, trypsin, periodate, amylase and extremes of pH (Baluyut *et al.*, 1981; Chang *et al.*, 1986; Sutherland and Redmond, 1986). The molecular weight was reported to range between 100 and 300 kDa (Himmel *et al.*, 1982; 1985; Mosier

et al., 1986; Chang *et al.*, 1986) and it is produced by all serotypable and non-serotypable strains (Shewen and Wilkie, 1983a; Sutherland and Donachie, 1986;). The former also showed that rabbit antiserum cross-neutralised the leukotoxin of different serotypes, indicating some homogeneity. Active growing cells of *P. haemolytica* have been shown to secrete leukotoxin, which can be cloned and produced using recombinant techniques. Strathdee and Lo (1987) have shown that the *P. haemolytica* leukotoxin genes and the corresponding protein share extensive homology with *E. coli* alpha haemolysin (50.3% of the amino acid residues are identical). Production of leukotoxin in the early stages of infection is important as it probably enables the bacterium to invade the alveolar macrophage. Neutralising antibodies have been demonstrated in the serum of sheep (Sutherland *et al.*, 1983) and are thought to be important in immunity.

2.2.5 Iron-Regulated Proteins (IRPs)

Some important immunogenic components such as iron-regulated proteins (IRPs) are expressed by cells *in vivo* and not by cells cultured on a complete medium *in vitro* (Morck *et al.*, 1991). Neilands (1982) have reported that these proteins are strongly expressed under conditions where iron availability is restricted as is the case in the animal host *in vivo* or in iron-depleted medium *in vitro.* Two IRPs of 70 and 100 kDa were present in the outer membrane of *P. haemolytica* A2 cells isolated directly from the pleural fluid of infected sheep (Donachie & Gilmour, 1988). These

proteins were expressed at a low level in A2 cells grown *in vitro* on iron-replete medium, but could be induced to a higher level of expression when the cells were grown in a medium which had been depleted of iron with an iron-chelating agent. Serum from convalescent sheep contained antibodies to both of these IRPs indicating that they were expressed in natural infection and were immunogenic. *P. haemolytica* IRPs are now known to bind ruminant transferrin and thereby obtain iron directly from the host system (Ogunnariwo & Schryvers 1990). Antibodies raised against IRPs may act immunologically by blocking the binding of transferrin leading to iron deprivation in the bacterium and thereby allowing the host to remove it.

2.2.6 Fimbrae

At least two types of fimbrae or pili, one thick and rigid and the other thin and flexible, were isolated from *P. haemolytica* serotype A1 grown in agar (Morck *et al.*, 1987; Potter *et al.*, 1987). These fimbrae can be isolated from bacteria grown under routine conditions for example in brain heart infusion broth at 37°C. Potter *et al.* (1987) reported that it may be possible to enhance fimbrial growth by subjecting various bacteria to elevated temperature or increased iron levels. The fimbrae are approximately 12 nm in diameter and vary in length from 100 nm to more than 500 nm. They may act by mediating adhesion to mucosal surfaces.

2.2.7 Neuraminidase

The majority of A serotype strains of *P. haemolytica,* but not the T serotype strains were found to produce the enzyme neuraminidase when grown overnight on blood agar (Frank and Tabatabai, 1981). Neuraminidase was initially found to be cell-associated, but Otulakowsky *et al.* (1983) demonstrated the enzyme in crude leukotoxin prepared from culture supernates. The significance of *P. haemolytica* neuraminidase in pathogenicity is unknown, but Gottschalk (1960) demonstrated that removal of the terminal sialic acid residue from salivary glycoprotein by the enzyme resulted in a loss of viscosity and adhesiveness, which could impair the protective function of the mucosal surface. The effect of this neuraminidase on A2 capsular polysaccharide is unknown.

2.2.8 Haemolysin

Haemolysin production may be one strategy used by *P. haemolytica* for acquiring iron from host red blood cells. Little is known about the haemolysin produced by the bacteria. Chang *et al.* (1987) demonstrated that its production does not appear to be plasmid mediated. It is generally believed that the leukotoxin is the haemolysin (Strathdee & Lo, 1987).

2.2.9 Protease

Otulakowsky *et al.* (1983) demonstrated that *P. haemolytica* produces a protease enzyme specific for the sialoglycoprotein on human red blood cells. This enzyme activity was demonstrated in most strains of *P. haemolytica* culture supernatant cytotoxic for bovine pulmonary macrophages.

2.3 Mouse model of disease

Experimental reproduction of sheep pasteurellosis is costly and the numbers of animals available is limited and seasonal. A model of the disease in laboratory animals would be advantageous, but unfortunately *P. haemolytica* is relatively non pathogenic for laboratory animals unless large numbers of organisms are used and thus increasing the possibility of endotoxin shock (Smith, 1959). Olitzki (1948) has described the use of mucin to enhance the virulence of organisms which are weakly or non-pathogenic when inoculated into mice. Smith (1959) developed two successful methods of infecting mice with *P. haemolytica*. One was an intra-cerebral inoculation of the bacteria suspended in hydrolysate solution which resulted in multiplication of the bacteria and death. The other method was by infecting the mice with a suspension of the bacteria in mucin intraperitoneally. This resulted in septicaemia and death of the mice within 48 hours. This model of the disease has proved to be most valuable and Smith (1959) used it to demonstrate passive protection and later active immunisation

of mice.

Biberstein and Thompson (1964) also used this model to investigate the roles of capsular and somatic antigens in immunity to *P. haemolytica.* The results suggested that capsular antigens play the major role in immunity with somatic antigens being of minor importance. However Knight *et al.* (1969) using the same model could not repeat the work and anomalies were found, one of these being that greater immunity was conferred by antigenically unrelated serotypes.

Cameron and Smit (1970) in trying to reproduce infection of mice in *P. haemolytica* using this method, also failed to get consistent results. A modification of Smith's original intra-peritoneal model was described by Evans and Wells (1979a) where after infection with *P. haemolytica* suspended in mucin, the multiplication of the bacteria was measured by counting the number of bacteria in the liver of the mouse. They obtained similar results to those found by Gilmour *et al.*, (1979) in sheep when testing sodium salicylate extract (SSE) vaccine of *P. haemolytica.* However in the experiment no protection was conferred against *P. haemolytica* serotype A2 challenge.

The reason for the enhancement of the virulence of organisms when mucin is given to mice are not fully understood and Calver *et al.* (1978) in his work with *N. meningitidis* has shown that the effect is due to the presence of iron in mucin. Others suggest that there is a simple protective role as a result of the viscosity of the mucin

suspension.

2.4 Sheep model of disease

Suitable systems to reproduce pneumonic pasteurellosis similar to the naturally occurring disease are important in order to study the interaction between the bacterium and the host. However, early efforts to reproduce the disease in sheep achieved only limited success (Salisbury, 1957). *P. haemolytica* is carried in the respiratory tract of high proportion of healthy sheep and in a survey into the prevalence of *P. haemolytica* in sheep Gilmour *et al.* (1979) isolated *P. haemolytica* from 95% of the sheep tonsils and from 64% of the nasopharynges. As a result, conventionally reared sheep have immune responses to *P. haemolytica* which may affect the consistent reproduction of the disease.

Studies by Shreeve *et al.* (1972) have shown that lambs experience colonisation quickly after birth probably as a result of intimate contact with their dams but this related to passive immunity. Smith *et al.* (1976) have shown that antibody titres in newborn lambs prior to suckling are generally low. Thus lambs deprived of colostrum might be expected to give reproducible results in challenge experiments. Gilmour *et al.* (1975) produced pneumonia indistinguishable from natural pneumonic pasteurellosis by exposing specific pathogen free (SPF) lambs to an aerosol of *P. haemolytica* serotype A1. SPF lambs are hysterectomy derived raised in microbiological secure

facilities (Hart *et al.*, 1971) and are free from *P. haemolytica.* Subsequent experiments showed that if SPF lambs are inoculated with both parainfluenza virus type 3 (PI_3) and *P. haemolytica* a high proportion of consistently animals develop clinical disease. (Sharp *et al.*, 1978). The success of the method of Sharp *et al.* (1978) allowed testing of vaccines in a controlled environment and Gilmour *et al.* (1979) reported successful immunisation of SPF lambs in a series of experiments using salicylate extracts (SSE) of *P. haemolytica.* Protection was demonstrated to be serotype specific, since a challenge with a serotype not included in the vaccine was successfully potent. This model of infection has proved useful for the study of *P. haemolytica* vaccines.

2.5 Vaccines and Immunity

Vaccination is generally the accepted means of control for pasteurellosis. Vaccines for most forms of the disease have been readily available throughout the world. Traditionally these have been plain bacterins containing killed bacteria cells of the particular *Pasteurella* species causing the disease. However the efficacy of the current crop of commercially available vaccines are on the whole, unsatisfactory (Gilmour, 1980). They do not induce good and strong immune responses required for protection against the disease. In an attempt to obtain a more satifactory vaccine, Gilmour *et al.* (1983) developed vaccines using cell extracts of serotypes A1, A6 and A9 which conferred a considerable degree of protection on SPF lambs against homologous, but not heterologous challenge. The results of similar vaccine experiments

with the A2 serotypes were not succesful. These limitations have stimulated continous research aimed at improving *Pasteurella* vaccines.

Advancement towards an effective *Pasteurella* vaccine, in particular against the A2 serotype, came from the finding that solid immunity against the A2 serotype was demonstrated when SPF lambs which had recovered from experimental pasteurellosis caused by serotype A2 were subsequently challenged (Donachie *et al.*, 1986). The sera from these recovered animals contained antibodies to a number of different virulence factors, namely OMP, IRP, leukotoxin, capsule and LPS. Subsequent serological studies in animals recovered from pasteurellosis showed a strong antibody response to these antigens, indicating their strong immunogenicity and their relevance as candidate antigens for vaccines. The protective efficacy of *P. haemolytica* IRP vaccine has been described by Gilmour *et al.* (1991). The identification and characterisation of other *in vivo* antigens is strongly indicated in all *Pasteurella* species for the improved antigenic composition of vaccines.

The incidence of the pneumonic pasteurellosis in Malaysia is increasing and there is continuing need for vaccines to be used for its control (Mohamad, unpublished 1993) However, the vaccines that are available to farmers are of questionable value (Wan Mohamad *et al.*, 1988) and since protection of sheep by *Pasteurella* vaccines are serotype-specific (Gilmour *et al.*, 1983) there is a possibility that the differences betwen the imported vaccine strains and the Malaysian field strains may result in this poor

efficacy. Therefore this thesis started with an epidemiological study to identify the serotypes of *P. haemolytica* involved in disease and to determine their relative prevalence in Malaysia. The findings emphasized the need for vaccine studies and Ovipast-9-IRP was tested for its efficacy in the field. Finally the importance of capsular antigen for a future formulation of improved *Pasteurella* vaccine was also determined.

CHAPTER 3.0 GENERAL MATERIALS AND METHODS

3.1 Bacterial Strains

All serotype strains of *P. haemolytica* used in this study were recovered from cases of sheep pneumonia in Malaysia and from the stock strains collection at Moredun Research Institute (MRI), Edinburgh. Scotland. These strains were subcultured into 50 ml of No.2 nutrient broth (Oxoid Ltd. Basingstoke, Hampshire) and grown for 18 h at 37°C without agitation after which 1 ml aliquots were put in 2 ml vials and stored at t -70°C until required.

3.2 Culture Conditions

When required, the bacteria were removed from -70°C storage, thawed and plated out on 7% sheep blood agar plates which were then incubated at 37°C for 18 h. Single colonies were picked from these plates and inoculated into 50 ml nutrient broths which were incubated at 37°C for 18 h. If required this was used as a seed broth for different types of culture media of larger volumes. Large quantities of bacterial cells

for extraction were produced by inoculating 5 litre volumes of different types of broth with 50 ml of seed broth. These were incubated at 37°C for 6, 18 or 24 h depending on the experimental requirements. For the 6 h culture the incubation was done on an orbital shaker (LH Engineering, Stoke Poges, Bucks). Bacteria were also grown for confluent growth on blood agar plates.

3.3 Preparation of *P. haemolytica* Antigens for Vaccines Trial

3.3.1 Preparation of crude Outer Membrane Protein-Polysaccharides (OMP-PS) complex

The main steps involved in this preparation are summarised in Figure 3.1. Preliminary purification was based on the method used by Moreno *et al.* (1985) for purifying outer membrane protein-polysaccharide complex from *N. meningitidis* Group B. Cells of *P. haemolytica* serotype A2 in 5 litre of nutrient broth culture incubated at 37°C for 6 h on an orbital shaker were removed by centrifugation for 1 h on an MSE coolspin centrifuge at 2500*g* (MSE, Crawley, Sussex). A 10% (w/v) solution of cetyltrimethyl bromide (Cetavlon) in water was immediately added to the culture supernatant at room temperature to give a final concentration of 1% (w/v). The mixture was left for 30 min at room temperature and the precipitate was collected by centrifugation at 10 000*g* for 20 min

at 4°C. The pellet was discarded and the supernatant was added to three volumes of absolute alcohol in an ice bath. The mixture was degassed under vacuum with swirling for 10 min and then left for 1 h at 0°C. The precipitate was collected by centrifugation at 10 000*g* for 20 min at 4°C, washed once in absolute alcohol and suspended in water (30 ml). The suspension was vortexed and placed in an ultrasonic bath (Beckman, UK) for 10 min. The mixture was centrifuged at 10 000*g* for 25 min at 4°C and the pellet was discarded. The changes in the reactions that occur in this preparation are shown in Plate 3.1.

3.3.2 Preparation of purified OMP-PS complex

The crude complex was further treated as described in Figure 3.2. Briefly the clear yellowish crude complex supernatant was chromatograhed by gel filtration on a column (2.6 cm by 100 cm) Sepharose CL-2B (Pharmacia, Uppsala, Sweden) equilibrated and run at 4°C with 0.1 M ammonium acetate pH 7 containing 0.01% (w/v) sodium azide.

Fractions (60 samples of 9 ml) were collected at a flow rate of 12 ml/h and analysed for absorbance at 260 and 280 nm and for sialic acid by the resorcinol hydrochloride method. The void volume fractions exhibiting absorbance at 280 nm and containing sialic acid positive material were pooled. The solution was sterilised by filtering through 0.45 μm and 0.22 μm millipore filter (Sartorius, Germany). Lactose

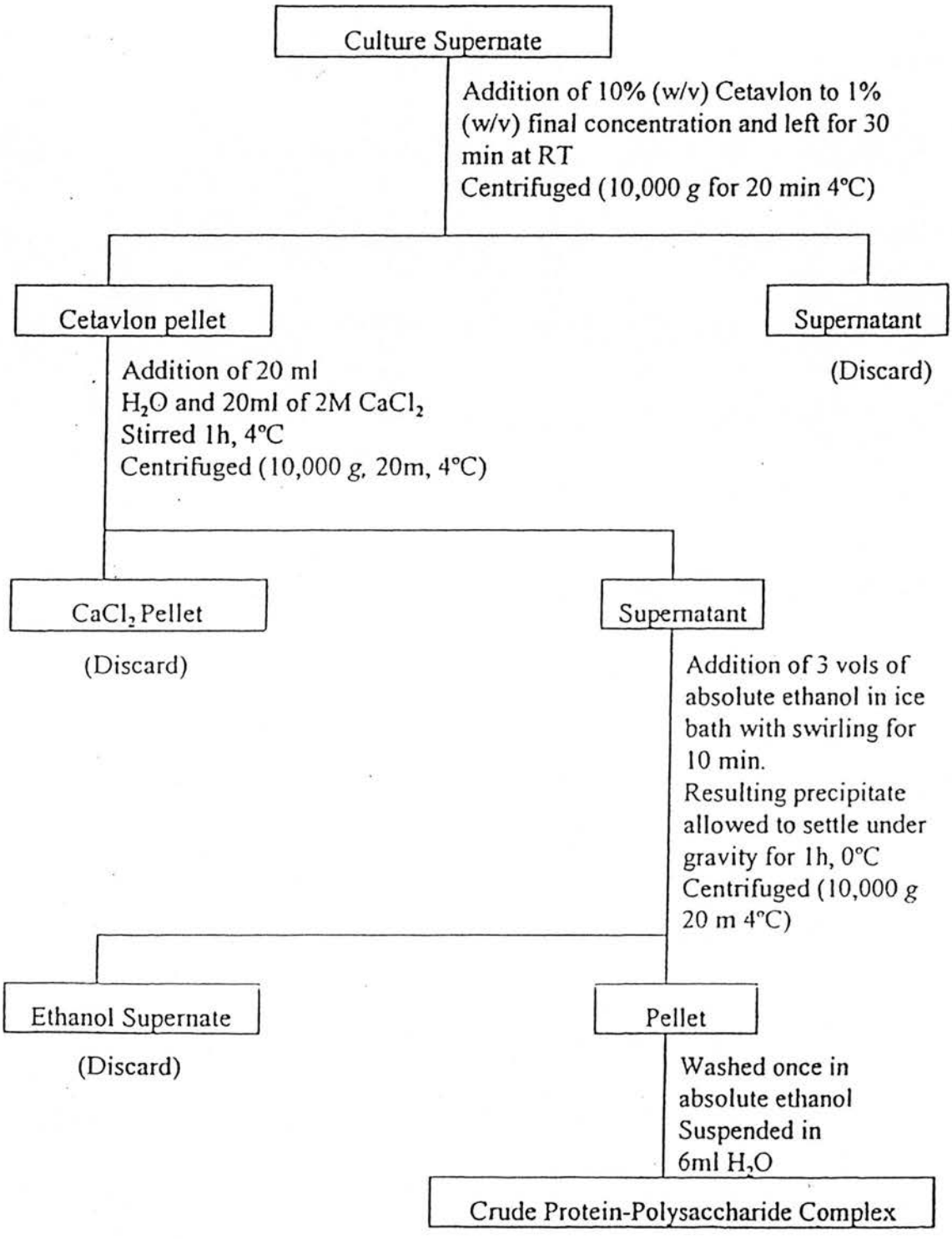

Figure 3.1. Preparation of crude outer membrane protein/polysaccharide complex of *P. haemolytica* A2

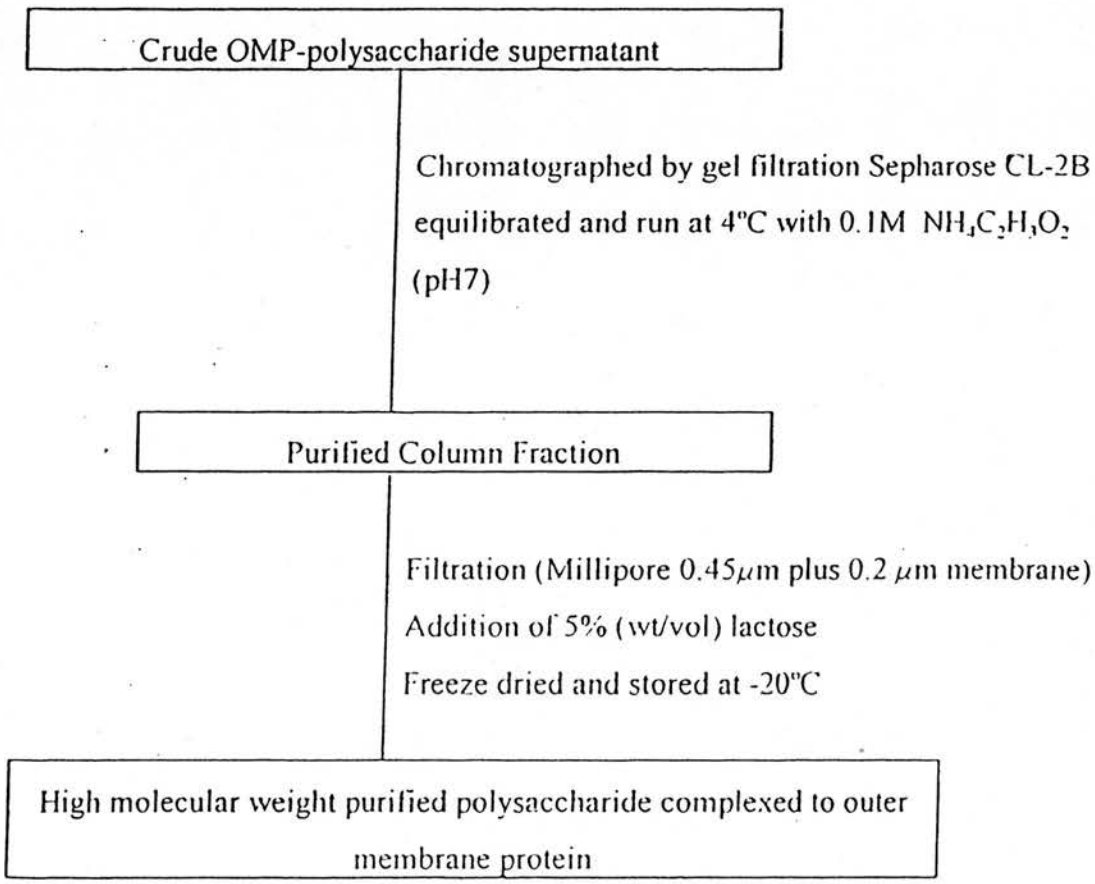

Figure 3.2. Further purification of outer membrane protein-polysaccharide complex of *P. haemolytica* A2

Plate 3.1. **Changes in the reactions of the materials during the preparation of capsular materials from *P. haemolytica* A2 by Cetavlon precipitation.**

Tube number

1 - **Starting A2 culture supernatant**
2 - **Culture supernatant + cetavlon (10% w/v)**
3 - **Cetavlon pellet**
4 - **Cetavlon supernatant + ethanol**
5 - **A2 pellet in ethanol**
6 - **Pellet suspended in H_2O**
7 - **Crude OMP-PS complex**
8 - **Purified OMP-PS column fraction**

5% (w/v) was added to stabilise the purified complex and then lyophilised until required (Tiesjema *et al.*, 1977).

3.4 Other vaccines preparations

3.4.1 Heated whole cells

P. haemolytica serotype A2 cells grown in 250 ml nutrient broth for 6 h at 37°C were heated at 56°C for 1 h and dialysed against distilled water before lyophilisation.

3.4.2 Washed whole cells

P. haemolytica A2 cells from 250 ml nutrient broth incubated at 37°C for 6 h were harvested by centrifugation at 4200*g* washed once in PBS and finally resuspended in 25 ml PBS and dialysed against distilled water for 24 h before lyophilisation.

3.5 Other vaccines used in the trials

Ovipast ^{R} **:** This was prepared by Hoechst, UK. It is comprised formalin-killed whole cells of *P. haemolytica* (serotypes A1, A2, A6, A7, A9 and T3, T4, T10, T15) grown under iron-restricted conditions. Aluminium hydroxide was added to the vaccines at 0.27% (v/v) final concentration.

Leukotoxin : Recombinant leukotoxin product. This was prepared by Dr. A. Lianson of MRI. Briefly leukotoxin A gene from *P. haemolytica* A1 was expressed in *E. coli* and partially purified by cell disruption and column chromatography (Lainson *et al.*, 1991).

Meningococcal Group B vaccine: This was kindly donated by Dr. R.Lifely (Wellcome Biotech, Kent, UK). The vaccine is a complex of capsule polysaccharide, outer membrane proteins and lipopolysaccharide suspended in alhydrogel. Typical composition of complex is 48.6% sialic acid, 46.8% protein and 4.1% LPS.

3.6 Antiserum production

Rabbit: Stock rabbit sera stored at MRI were used for serotyping of *P.haemolytica*. Rabbits were immunised with repeated doses of formalin-killed cells of *P. haemolytica* according to the following schedule:

A subcutaneous inoculum of 0.5 ml folllowed by intravenous doses 1 ml, 2 ml and six doses of 3 ml at intervals of three to four days were administered to the rabbits. A trial bleed was made ten days after the last inoculation and if the titre was satisfactory, the rabbit was exsanguinated by cardiac puncture. Sera were stored in small aliquots at -20° C for up to 2 years.

3.7 Serology

3.7.1 Indirect haemagglutination test (IHA)

Serotyping: *P. haemolytica* isolates were serotyped using a modification of the test first described by Biberstein (1978), Fraser *et al.*, (1982). *P. haemolytica* isolates were subcultured onto blood agar plates to check purity and to maintain the strain. Growth from blood agar, preferably several colonies, was suspended in 1 ml of formalised phosphate buffered saline (FPBS) (0.3% formalin) in a microcentrifuge tube. The bacterial suspension was heated in a 56°C water bath for 30 min to release the antigen from the cells after which bovine RBCs, fixed in 1% glutaraldehyde (Shirai *et al.*, 1975) were added to a final concentration of 0.5% and incubated at 37°C for 30 min. The sensitised RBCs were pelleted at 2900*g* and washed three times in FPBS and then resuspended in 1 ml FPBS. One drop (0.025 ml) of each of the 16 diluted rabbit antisera was dispensed into U-bottom microtitre plates (Cookes Engineering Company, Alexandria, Virginia, USA). After addition of 0.025ml of sensitised RBCs suspensions to the appropriate wells the plate was left at room temperature for 2 h or overnight before examination for haemagglutination. A positive result was indicated by an even mat of RBCs over the bottom of the well, while negative results were indicated by a small button of RBCs in the centre of the well.

3.7.1.1 Measurement of antigen

The samples to be tested were diluted in FPBS in ten fold dilution from 1 in 10 to 1 in 100,000 in 2 ml volumes. Glutaraldehyde fixed RBCs were sensitised by adding to the sample 0.2 ml of a 5% washed suspension of RBCs. Cells were washed as described above then added in 0.025 ml volumes to microtitre plate wells in duplicate. Equal volumes of positive or negative control sera were then added and the highest dilution of sample which produced haemagglutination was the IHA titre of the sample.

3.7.1.2 Measurement of antibody

Antibodies against *P. haemolytica* were measured by testing serum samples by the IHA method described by Shreeve *et al.*, (1972) and modified by Fraser *et al.*, (1983).

3.7.2 Enzyme-linked immunosorbent assay (ELISA)

The technique used was basically that described by Burrells *et al.* 1979).

3.7.2.1 Indirect ELISA for detecting 35 kDa antibody

The antigen, *P. haemolytica* A2 35 kDa IRP prepared by affinity chromatography, was diluted in carbonate/bicarbonate buffer, pH 9.6, to give a final concentration of 200 ng/ml and added in 100 μl volumes to wells in a microtitre plate (Dynatech 129A, Laboratories Ltd. Billinghurst, Sussex). The plates were incubated at 4°C overnight. Plates were then washed three times with PBS containing 0.05% Tween 20 (PBS/Tween). Horse serum (20%) was used to avoid non specific adsorbtion. Standard and test sera were added to 100 μl volumes to the wells and incubated at 4°C overnight. The standard positive convalescent sera was titrated at 1/50-1/6400 dilutions, while the negative and test sera were used at a dilution of 1/50. Serum dilutions were removed from the wells, the plates washed three times with PBS/Tween and 100 μl of donkey anti-sheep IgG conjugated with horse-radish peroxidase (HRP) diluted 1/200 in PBS/Tween added to the wells. After incubation for 1 h at 37°C the plates were emptied and washed. Enzyme substrate {orthophenylamine diamine (OPD), Sigma Chemical Co., Poole, Dorset} at a concentration of 0.04% in citrate phosphate buffer pH 5 was added to the plates in 100 μl volumes. After 5 min at room temperature the reaction was stopped by addition of 50 μl of 2.3 M sulphuric acid. Results were recorded as optical densities at 492 nm (OD_{492}) and were determined using a multichannel spectrophotometer (Dynatech 5000).

3.7.2.2 Sandwich ELISA for detecting anti-A1 capsule antibodies

Flat bottomed micro-ELISA plates were coated with a 1/100 dilution of anti-A1 capsule monoclonal antibody (7/13) (Wilson *et al.*, 1991) at 37°C overnight. Plates were then washed and 50% horse serum in PBS was added as a blocking step and incubated for 1 h at 37°C. The plates were washed three times with PBS/Tween and A1 capsular antigen at 1/50 dilution in PBS/Tween were added to wells and incubated for 90 min at 37°C. The capsular antigen was prepared by heating 1 litre of 18 h culture at 56°C for 1 h. Cells were removed by centrifugation and filtration before lyophylisation. Before using the freeze dried material was re-suspended in 4% of the original volume. The remaining steps are as described in section 3.7.2.1.

3.7.2.3 ELISA to detect *P. haemolytica* A2 OMP antibodies

The OMP were prepared as described by Donachie & Gilmour (1988). Briefly, washed cells were sonicated in an MSE sonicator (150 watt, MK2) for three pulses at maximum power with intermittent cooling. The sonicate was then centrifuged at 4000*g* and the supernatant fluid centrifuged at 30,000*g* for 1 h to pellet envelopes. The envelopes were re-suspended in PBS with 2 % Sarkosyl (w/v), incubated at 37°C for 1 h and then centrifuged at 4°C at 100,000*g*. The pellet was resuspended in 0.01M TRIS. This OMP diluted in carbonate/bicarbonate buffer of pH 9.6 was used to coat the micro-ELISA plates. The remaining steps of the ELISA were as described above for 35

kDa IRP ELISA.

3.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A discontinuous system employing slabs of gel 1.5 mm thick with a 12% (w/v) separating gel and a 4% stacking gel and the buffer system of Laemli (1970) was used. Samples were diluted 1:1 with sample buffer containing 2% (w/v) SDS and 4% (v/v) 2-mercaptoethanol and boiled for 5 min before application to the gels. The gel was run for approximately 6 h with the voltage set at 350 volts. Proteins were detected by Coomassie-blue or silver staining. LPS was detected by the silver staining method of Tsai & Frash (1982).

3.9 Western blotting

The method of Burnette (1981) as modified by Sharp & Herring (1983) was used. Electrophoretically resolved proteins were transferred on to nitrocellulose paper (pore size 0.45 μm, Sartorius) at 0.11 A for 6 h or overnight at RT. The nitrocellulose sheet was stained with Ponceau red for 30 sec at room temperature to make sure the protein had been successfully transferred onto nitrocellulose. The sheet was then immersed in horse serum in an equal volume of blot wash buffer {BWB, PBS with 0.5M NaCl, 0.5M 0.001 M-EDTA and 0.5% (v/v) Tween 20} and shaken on an orbital shaker for 1 h at 37℃. The fluid was discarded and replaced with horse serum in BWB

(1:9 v/v) containing the serum (2.5% v/v) to be tested. When different sera were to be tested the nitrocellulose sheet was cut into 5mm strips and placed in a blotting tray (BioRad). Two millilitres of diluted test sera were added to each well. Positive and negative controls were also included. After incubation at 37°C for 1 h, the sheet was washed in BWB. Subsequently, the sheets were incubated with horse-radish peroxidase (HRP) conjugated anti-mouse or anti-sheep Ig {Sigma or Scottish Antibody Production Unit (SAPU) } and then again extensively washed as before. Colour development was carried out at room temperature in 0.1M Tris buffered saline (TBS) (pH 7.6) containing diaminobenzidine (DAB) at 0.5 mg/ml and 2 μl of 30% (v/v) H_2O_2 The sheets were washed in tap water to stop any further enzyme substrate reaction. The immunoblots were dried and analysed for antibody/antigen reactions.

3.10 Dot blotting

The dot blot apparatus manufactured by Millipore, UK was used. Nitrocellulose paper was set in the apparatus and the test antigens added to the wells and left to bind for 1 h at 37°C. The nitrocellulose sheet was then removed and the whole sheet blocked with 20% horse serum. The sheet was then exposed to appropriate antisera in 5% Marvel (Primary Beverages, Stafford, UK) in PBS. After incubation at 37°C for 1 h the sheet was washed in BWB. Sheep anti-mouse IgG conjugated to HRP (SAPU) (1:200 in BWB) was added and incubation carried out for 1 h at 37°C. After washing, the substrate solution {(5mg DAB in 20 ml of Tris pH 7.4) in 50 μl H_2O_2} was added until

colour developed. The reaction was stopped with distilled water and the blot was analysed.

3.11 Chemical analysis

3.11.1 Protein

Protein in the test sample was detected and measured by the techniques and procedure described in the Pierce BCA Protein Assay Method. Briefly 200 μl working reagent (Pierce BCA Protein Assay Reagent) was dispensed into wells of microtitre plates. After the addition of 25 μl of each standard or unknown protein sample into each well the plate was incubated for 30 min at 37°C. The plates were cooled to room temperature and the absorbance at 562 nm was measured. The protein concentration of the sample was calculated using a prepared standard curve.

3.11.2 Sialic acid

Quantitative determination of sialic acids was achieved by the method of Svennerholm (1979). Briefly the reagent was added to test samples and the tubes heated for 15 min in boiling water bath. The tubes were cooled and amyl alcohol added. The absorbance at 450 and 580 mμ were read against pure amyl alcohol as blank using a

Beckman Spectrophotometer. The amount of sialic acid was calculated using a prepared standard curve.

3.11.3 Lipopolysaccharides (LPS)

LPS content was determined by the kinetic turbidimetric assay method using Limulus Amoebocyte Lysate (LAL) as described by Harris *et al.* (1983). Sequential readings at absorbance of 380 nm, were taken on an IL Monarch Centrifugal Analyser.

3.12 Experimental animal techniques

Mice: Mice used in experiments were C57 black strain, bred at the MRI. The mice were of both sexes and were 6-8 weeks old at the start of the experiment.

Inoculations : Intraperitoneal (i.p.) injections into mice were given without anaesthesia. Mice were injected into the abdomen slightly to the left of the umbilicus using a 25 gauge needle.

Mouse model of *P. haemolytica* infection: The model described by Evans and Wells (1979a) was adopted. Groups of mice were inoculated i.p. with at least 2 LD_{50} of the

P. haemolytica isolate required in hog gastric mucin (ICN Pharmaceuticals). After 6 h the mice were killed by cervical dislocation and the livers removed aseptically. These were then individually macerated with 9 ml of peptone water (Oxoid) in a Colworth stomacher

(Seward, London). Serial ten-fold dilution in peptone water were plated out for counting of viable cells by the method of Miles *et al.* (1938). The mean viable count and standard errors of the means each group was calculated and expressed as $\log_{10}$.

3.13 Vaccines formulation

Antigen and vaccine used were resuspended in distilled water to the required dosage concentration. Aluminium hydroxide (Alhydrogel, Superfos Denmark) was added to all vaccines to a 0.27% (v/v) final concentration.

Vaccination and challenge of mice: Ten mice of various groups were each given two doses of 0.1 ml of vaccine intraperitoneally with 14 days between the two inoculations. Fourteen days after the second inoculation, these mice and a group of uninoculated control mice, were challenged i.p. as described in the mouse infection model. After 6 h the mice were killed and viable counts performed on the whole liver suspension.

Vaccination and challenge of sheep: Three groups of 4 lambs aged 3 months and 3 groups of ewes aged 2 years were used in this experiment. The sheep had been clinically

healthy and tested negative for *Pasteurella spp.* The ewes and the lambs were randomly allocated according to vaccines group. Two vaccine preparations consisting of crude and purified OMP-PS vaccines complex were administered to two groups in each age group. The animals were vaccinated subcutaneously (2ml dose) on the lateral aspect of the middle third of the neck at day 0 and booster given 4 weeks later. The remaining group in each group were sham-vaccinated and served as controls. The animals were grazed together at pasture raised at Moredun farm for the duration of the experiment.

3.14 Statistical analysis

Where possible, t test were performed and means and SE were calculated. Where the data were unsuitable, the Mann-Whitney Ranking Test (Snedecor & Cochrane, 1987) was used to determine the significant differences between group data.

CHAPTER 4.0 EPIDEMIOLOGICAL STUDIES ON *PASTEURELLA HAEMOLYTICA* IN MALAYSIAN SHEEP AND GOATS

4.1 Serotyping of Malaysian *P. haemolytica* isolates

4.1.1 Introduction

An epidemiological study of pasteurellosis in Malaysian sheep and goats is required to identify the serotypes of *P. haemolytica* involved in disease and determine their relative prevalence. This information can then be used to develop relevant control programmes involving management and disease prevention methods. As immunity to *P. haemolytica* infections is believed to be serotype specific this type of study should also help identify the serotypes to be included in vaccines.

There have been no reports on the prevalence of different serotypes in the country and the first study was initiated in 1989 where strains of *P. haemolytica* were sent to the Moredun Research Institute (MRI) for serotyping. The purpose of

this study was to to determine the range of serotypes isolated during the year 1989-1993 from sheep and goats in Malaysia.

4.1.2 Materials and Methods

The strains submitted were isolated at Malaysian Agriculture Research and Development Institute (MARDI), University Pertanian Malaysia, the Regional Diagnostic Laboratory and the Veterinary Research Institute from sheep and goats throughout Malaysia, which were either clinically abnormal or had a pathological condition at post mortem (see map in Appendix I).

Strains were received on blood agar plates or slopes or freeze-dried and were serotyped either directly from the original culture or after subculture on 5% sheep blood agar. Strains were serotyped by the rapid IHA test as described in the general Materials and Methods of this thesis.

4.1.3 Results

A total of 437 *P. haemolytica* strains was submitted for serotyping (320 isolates from sheep and 117 from goats) and 364 typable (287 from sheep and 77 from goats) and 73 untypable (33 from sheep and 40 from goats) strains were isolated. The majority of sheep and goats isolates were from lambs and kids (73% and 71% respectively). The *P. haemolytica* isolates in these animals were primarily associated with pneumonia in adult animals and septicaemia in the young. The predominant

serotypes were A2, A1, A9 and A7 (38.2%, 13.7%, 13% and 10.1% respectively). Six A serotypes (A5, A6, A8, A11, A12 and A13) were isolated in smaller numbers. The isolates of biotype T was very rare and only serotype T3 (2.5%) was isolated (Table 4.1). The serotype distribution pattern is quite similar for both sheep and goats.

The clinical disease condition from which *P. haemolytica* strains were derived and the main pathological conditions diagnosed are shown in Tables A & B in the Apppendix II. The organ from which *P. haemolytica* strains were isolated are shown in Table C. Complete clinical and pathological data for the cases suspected of pasteurellosis submitted to the various regional diagnostic laboratories were not available for all the laboratories. In post mortem examinations of animals only lungs and lymph nodes were normally examined and cultured for the bacterial isolation. In this study eighty percent (351/437) of the strains were isolated from lungs. Recovery of the organisms from nasal swabs was very poor (only 25 cultured out of 124 samples submitted). *P. haemolytica* were also recovered from the other organs but only account for 14 % (61/437) of the total strains isolated.

Histopathological examination was performed on lungs from majority of sheep and goats cases. *P. haemolytica* strains were associated mainly with fibrinous pneumonia and bronchopneumonia. The complete histopathological data were only available from two centres in Petaling Jaya and University Pertanian Malaysia

laboratories. Untypable strains comprised 16.7% of the isolates. Proportionally there was a higher incidence of these strains in goats (54.8%) than in sheep (45.2%). Other bacteria were also isolated from 25% (108/437) of the animals from which *P. haemolytica* strains were also isolated. From pneumonic lungs *P. multocida, Haemophilus somnus* and *Actinobacillus* spp. were isolated. *Clostridium* spp., *Salmonella* spp., *E. coli* and *Listeria monocytogenes* were recovered from septicaemic cases.

Table 4.1. The prevalance of serotypes of *P. haemolytica* isolated from sheep and goats pasteurellosis in Malaysia during 1989-1993.

Serotypes	1989*	1990	1991	1992	1993	1989-93	Nos
A1	7	11.5	28	11	11.5	13.7	60
A2	14	36.5	55	44.5	38	38.2	167
A5	7	0	0	2	0	1.8	8
A6	7	0	0	2	1	2	9
A7	11	14.5	8	11	7	10.1	44
A8	0	0	0	1	3	0.8	3
A9	36	7.5	0	14	8	13	57
A11	0	2.5	0	0	0.5	0.6	2
A12	0	0	0	1	1.5	0.5	2
A13	0	0	0	0	1	0.2	1
T3	7	0	0	2	3.5	2.5	11
UT	11	27.5	9	11.5	25	16.7	73
Total Nos	80	50	85	112	110		437

*-Expressed as a percentage of the total number of strains examined.

Table 4.2. The distribution of serotypes of *P. haemolytica* isolated from cases of ovine pasteurellosis in United Kingdom, 1982-1993

Serotypes/%	1982*	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1982-93	Nos
A1	4.7	6.4	6.0	6.7	6.1	5.8	4.9	3.0	3.0	7.7	6.1	3.2	5.9	511
A2	33.0	32.0	39.5	32.6	29.0	33.2	42.4	39.5	32.9	28.6	15.5	18.9	33.9	2959
T3	2.0	3.5	2.0	2.0	3.0	2.9	4.4	2.6	2.6	2.4	6.1	3.3	2.6	226
T4	8.1	8.2	6.3	6.9	7.8	6.6	2.6	6.1	6.6	8.4	9.4	9.0	7.0	612
A5	0	0.7	0.5	1.0	1.0	0.5	0.9	0.4	0.7	0.3	0	0.8	0.6	55
A6	8.1	5.8	5.7	7.5	7.5	4.4	8.1	4.2	5.0	4.9	2.2	2.5	6.2	542
A7	4.9	4.1	5.3	5.4	5.0	2.9	4.4	7.2	1.7	1.8	3.3	1.6	4.6	401
A8	0.5	0.3	1.3	1.3	1.4	0.7	0.3	0.4	0.7	0.3	0	1.6	0.9	79
A9	3.7	5.1	5.1	5.5	3.7	3.7	3.5	3.8	5.3	4.2	0.5	2.5	4.6	401
T10	12.9	14.0	9.1	9.3	9.1	9.9	9.0	10.3	14.0	21.6	21.0	22.1	11.3	985
A11	2.4	2.6	1.9	3.0	4.0	4.0	1.7	0.8	2.6	0.3	0.5	0.8	2.5	215
A12	2.7	1.9	2.7	2.0	1.2	2.7	3.5	1.9	2.0	0.7	0.5	3.3	2.2	195
A13	1.0	0.7	1.1	1.3	2.3	1.4	0.3	0.4	0.3	0.7	0.5	2.5	1.1	97
A14	0	0	0	0.2	0.2	0	0	0	0	0	0	0	0.05	4
T15	10.1	10.1	6.9	6.1	10.5	8.9	5.2	11.4	13.6	7.3	27.1	20.5	8.8	767
A16	x	x	x	x	x	0	0	0	0	0	0	0	0	0
UT	5.9	4.6	6.6	9.1	8.2	12.5	8.7	8.0	9.0	10.8	7.2	7.4	7.7	678
Total Nos Typed	801	1213	2049	1864	573	729	344	263	301	287	181	122		8727

* - Expressed as a percentage of the total number of strains examined.
x - Not done.

4.1.4 Discussion

Not all laboratories record diagnoses in the same manner so some interpretation had to be applied in order to summarise the information. Nevertheless, the *P. haemolytica* isolates recorded in this study are considered to be a representative sample of the prevalence of this organism in the small ruminant population in Malaysia. Although complete clinical and pathological data for the disease were not available for all the laboratories, the majority of the cases had undergone post mortem examination and gross pathology and histopathology results were available for most of the cases and can be regarded as conclusive of pasteurellosis. A small number of isolates were from nasal swabs and the problem of poor isolation and sample contamination were unavoidable. The application of long swabs to disinfected nasal region partly solved the problem.

The primary diseases associated with *P. haemolytica* were pneumonia in adult sheep and goats and septicaemia in the young, as has been extensively recorded elsewhere (Gilmour *et al.*, 1980). The serotypes isolated from these conditions also largely agree with the recognised pattern in sheep (Gilmour & Gilmour, 1989) with serotype A2 being the most important pneumonic isolate in sheep and goats. However, T biotypes were rare in Malaysian isolates probably because they are associated with temperate climate animals (Gilmour & Gilmour, 1989). Of the 437 isolates of *P. haemolytica* typed 16.7% were untypable. Similar to results of this was recorded by

Biberstein and Thompson (1976) where 12% and 28% of nasal isolates were untypable in two surveys and Ball *et al.* (1993) reported that 18% of their isolates were untypable.

The present study indicate that the serotype distribution for the A serotypes is quite similar and not significantly different ($P>0.05$) to a survey of ovine pasteurellosis cases at MRI during 1982-1993 (Table 4.2) (Quirie, 1994, unpublished data) and in Northern Ireland (Ball *et al.*, 1993), indicating little variation in this distribution in Malaysia.

These results may be of significance in the formulation of vaccines against ovine and caprine pasteurellosis in Malaysia, as they give an indication of the serotypes most commonly found in pneumonic cases in the field.

4.2 OMP and LPS profiles of Malaysian and United Kingdom strains - a comparison

4.2.1 Introduction

Ovine pneumonic pasteurellosis has caused serious economic loss to the farming industry of Malaysia and the United Kingdom. In Malaysia the disease remains a very serious problem accounting for more than 50% of sheep and goats mortalities despite the use of vaccines. The vaccines commonly used in Malaysia are imported from the UK and although they contain the common important serotypes, much poorer efficacy was experienced in Malaysian farms (Wan Mohamad *et al.*, 1988). From the above serotyping studies it was shown that the serotype distribution of *P. haemolytica* for the A biotype strains for Malaysia and the United Kingdom are quite similar. It is possible that the differences which might be present between the serotypes from both countries possibly contribute to this poor protection of the animals.

There are 17 immunologically distinct serotypes of *P. haemolytica*. Vaccination with one serotype does not confer protection on animals against challenge with another serotype (Gilmour *et al.*, 1983). In addition, antigenic differences within the serotypes may be such that vaccination with one strain may not give complete protection

against another strain of the same type (Donachie, personal communication). More recently the ELISA has been used for studying antigenic relationships and vaccine strain selection among pathogenic organisms. Differences between strains have also been shown by non-serological methods. Analysis of outer membrane protein (OMP) and lipopolysaccharide (LPS) profiles has been used for examining strain variation within species of bacterial pathogens. Typing scheme based on OMP and LPS profiles in SDS-PAGE have been developed and used for epidemiologiacal studies in both human and veterinary pathogens (Loeb and Smith, 1980; Lugtenberg *et al.*, 1984; Ali *et al.*, 1992). The combined analysis of both OMP and LPS profiles by SDS-PAGE has also been carried out for *E. coli* (Achtman and Plushchke, 1986) and *P. multocida* (Lugtenberg *et al.*, 1984).

The OMP and LPS of *P. haemolytica* have not been studied widely. Little is known about variation of OMP and LPS within the species particularly within biotype A strains. Although the LPS from different serotypes is known to be antigenically related (Donachie *et al.*, 1984b; Durham *et al.*, 1988)), major differences occur in the LPS associated with A and T biotypes. Adlam, (1989) reported strains belonging to A biotypes have a characteristic rough-type LPS whereas T biotypes are of smooth-type. When analysed by SDS-PAGE and silver staining, smooth-type LPS is separated into low molecular mass fraction, consisting of the lipid A-oligosaccharide core region and a distinctive ladder pattern of high molecular mass bands while the rough LPS consisting

only of the core oligosaccharide lipd A visualised as a number of low molecular mass bands only (Hitchcock *et al.*, 1986).

The present study was conducted to examine and compare the OMP and LPS profiles of a selection of common serotypes of *P. haemolytica* isolates obtained from sheep pneumonia in the UK and Malaysia, with the aim of identifying similarities and differences within the serotypes which could be of epidemiological significance or prove useful to formulate more effective field vaccines.

4.2.2 Materials and methods

Bacterial strains: Twenty four isolates of *P. haemolytica* common to UK and Malaysia were examined in the present study. The isolates comprised of 6 each of serotype A1, A2 A7 and A9 including four from healthy sheep and the remainder from pneumonic cases of sheep and goats. Isolates from healthy sheep were obtained from the nasopharynx whereas those from pneumonic animals were obtained from the lung lesions.

The isolates from Malaysia were obtained from the farms mentioned earlier in Chapter 4 and were made over a three year period (1990-93). The UK isolates were obtained from the reference stock strains collections at Moredun Research Institute. The

Table 4.3. *Pasteurella haemolytica* strains examined in the study

Strain	Serotype	Source of strains		
		Origin	Host species	Site of isolation
M 954/89	A1	Malaysia	Ovine	Pneumonic lung
M/M 1.2/92	A1	Malaysia	Ovine	Nasal cavity (healthy)
M/M 1.3/92	A1	Malaysia	Caprine	Pneumonic lung
M/M 2.1/92	A2	Malaysia	Ovine	Nasal cavity (healthy)
M 945/89	A2	Malaysia	Ovine	Pneumonic lung
M/M 2.2/92	A2	Malaysia	Caprine	Pneumonic lung
M/M 7.1/92	A7	Malaysia	Caprine	Pneumonic lung
M 927/89	A7	Malaysia	Ovine	Pneumonic lung
M/M 7.2/92	A7	Malaysia	Ovine	Nasal cavity (healthy)
M/M 9.1/92	A9	Malaysia	Caprine	Pneumonic lung
M/M 9.2/92	A9	Malaysia	Ovine	Nasal cavity (healthy)
M 933/89	A9	Malaysia	Caprine	Pneumonic lung
H 379	A1	UK	Ovine	Pneumonic lung
DO 151	A1	UK	Ovine	Pneumonic lung
H183	A1	UK	Ovine	Pneumonic lung
EO 200	A2	UK	Ovine	Pneumonic lung
DO 152	A2	UK	Ovine	Pneumonic lung
DO 689	A2	UK	Ovine	Pneumonic lung
DO 123	A7	UK	Ovine	Pneumonic lung
H 35	A7	UK	Ovine	Pneumonic lung
H 36	A7	UK	Ovine	Pneumonic lung
DO 148	A9	UK	Ovine	Pneumonic lung
H 49	A9	UK	Ovine	Pneumonic lung
H 185/6	A9	UK	Ovine	Pneumonic lung

details are shown in Table 4.3. After primary isolation, the isolates were stored in nutrient broth No. 2 (Gibco) at -70°C and were routinely subcultured on blood agar (Oxoid) containing 5% sheep blood at 37°C.

Preparation of whole cells, envelopes and OMP: Bacterial whole cells, envelopes and OMP were prepared as described by Donachie & Gilmour (1988). When required 50 ml seed cultures were intiated in nutrient broth (Gibco No.2) and six hours later inoculated into 1 litre volume. Cells were grown at 37°C overnight, then harvested by centrifugation and washed twice in PBS (0.01 M-sodium phosphate, 0.15M-NaCl, pH 7.4). Bacterial suspensions were adjusted to give protein concentration of 1.5 mg ml^{-1} as determined by Pierce BCA Protein Assay Reagent. The washed cells were sonicated in an MSE sonicator (150 watt, MK2) for 30 second pulses at maximum power with intermittent cooling. The sonicate was then centrifuged at 40 000 *g* and the supernatant fluid centrifuged at 30 000 *g* for 1 h to pellet envelopes. Envelopes were suspended in 0.1 M Tris/HCl, pH 7.4. The pellet was then resuspended in 4 ml PBS and Sarkosyl (2% w/v) was added. The suspension was incubated at 37°C for 1 h and harvested by centrifugation at 100 000*g* for 1 h. The OMP pellet was resuspended in 50 l Tris/HCl (0.1M, pH7.4) before storage at 20°C.

Preparation of LPS: LPS was obtained by a modified method as

described by Donachie (1984a). Briefly bacterial colonies from an agar plate were taken and suspended in 45% aqueous phenol in a microcentrifuge tube. The suspension was agitated vigorously for 10 min then cooled on ice at 4°C for 30 min. followed by centrifugation at 3500 g for 10 min. The top layer which contained the LPS was removed and loaded directly in the gels.

SDS-PAGE: *P. haemolytica* proteins were separated by SDS-PAGE, using a modification of the method described by Laemli (1970) as described in the general Materials and Methods.

4.2.3 Results

The Protein Profiles

The results of PAGE are shown in Figure 4.1 and Figure 4.2. The OMP profiles of strains from UK and Malaysia show no distinct differences. Different isolates of the same serotypes had similar polypeptide profiles (Fig. 4.1 Lane 2-9). The reference A2 strain from UK and field A2 strain from Malaysia showed slight differences in migration of structural polypeptides (Fig.4.1, Lane 4 and 5). The patterns for the other serotypes, A1, A7 and A9 (Fig.4.1, Lane 2, 3, 6, 7, 8 and 9) were very similar.

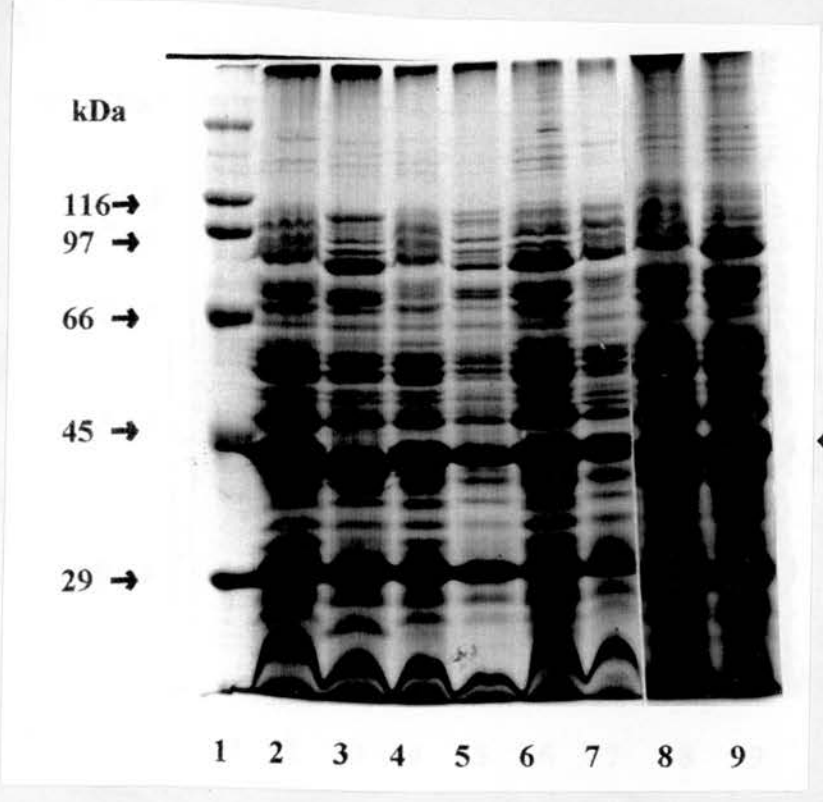

Figure 4.1. SDS- PAGE of whole cells proteins *P. haemolytica* serotypes A1, A2, A7, A9 common to Malaysia (Lane 2, 4, 6 and 8) and the UK (Lane 3, 5, 7 and 9). Molecular mass markers are shown in lane 1. Each lane contained approx. 20 μg of protein.

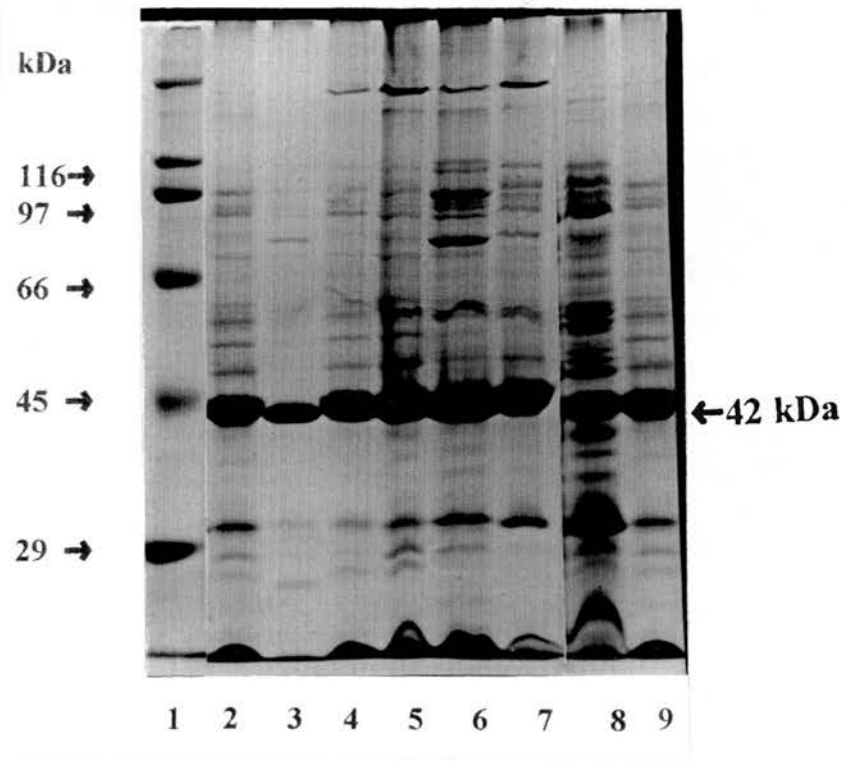

Figure 4.2. SDS- PAGE of envelope proteins *P. haemolytica* serotypes A1, A2, A7, A9 common to Malaysia (Lane 2, 4, 6 and 8) and the UK (Lane 3, 5, 7 and 9). Molecular mass markers are shown in lane 1. Each lane contained approx. 20 μg of protein.

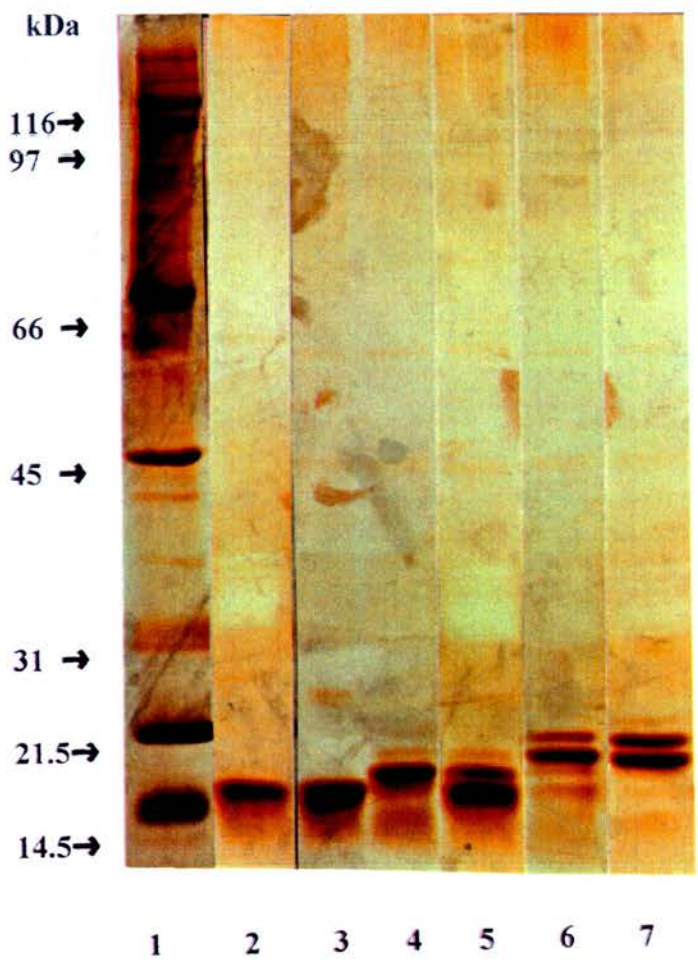

Figure 4.3. **SDS-PAGE of *P. haemolytica* LPS of serotypes A1, A2 and A7 common to Malaysia (Lane 2, 4 and 6) and the UK (Lane 3, 5 and 7). The LPS obtained by rapid aqueous-phenol method and silver-stained. They are of the rough type 3 LPS.**

The OMP profiles in Coomassie-blue-stained gels, of serotype A2 from both locations were very similar and consisted of five major proteins and approximately 17-21 minor proteins. The profiles of the serotype A1, A7 and A9 from both locations were similar. The OMP fractions which could be assumed as major proteins are of apparent molecular masses of 29,39,40 and 42 kDa.

The LPS profiles

In the present study, the LPS profiles of 18 isolates of *P. haemolytica* from both countries comprising of serotypes A1, A2 and A7 were examined. Analysis of LPS revealed 3 distinct profiles among the A biotype. Representative examples of these LPS profiles are shown in Figure 4.3. It was noted that the resolution of the high molecular mass region is poor. The LPS pattern among the serotypes A1 and A7 appeared to be identical and do not have as many bands as the ladder-patterns of the smooth-type LPS of the T biotype (results not shown). Comparison of the LPS patterns of the A2 strains from UK and Malaysia showed a difference at 18 kDa region but both are rough types.

4.2.4 Discussion

The objective of this study was to examine and compare the OMP and LPS profiles of a selection of common *P. haemolytica* isolates obtained from Malaysian and UK sheep farms. The aim was to detect any differences which could be useful in epidemiological studies and later help in the formulation of universal vaccines. The OMP profiles of 24 isolates examined in the SDS-PAGE gels were similar to those described in a previous study of the same A serotypes from UK isolates (Murrray, 1992, unpublished data).

The reference and the field isolates strains from the two countries showed slight differences in migration of structural polypeptides but were generally very similar. The field strains showed a gel profile in which all the major polypeptides migrated quite similarly to all of the reference strains used in the study. This findings are in agreement with Knights *et al.* (1990) who showed that considerable qualitative similarities were noted within the A biotype isolates.

The OMP profiles comprised of four major proteins with apparent molecular masses of 29, 39, 40 and 42 kDa and between 17-21 minor proteins. This findings are with agreement with that of Davies *et al.* (1992) who compares the OMP

profiles of 2 isolates of serotype A1 demonstrated that four to five major proteins with at least twenty minor proteins were present. The authors also suggested that the molecular masses of the major proteins are located within the same molecular mass range desribed.

The results also confimed that obtained by Squire *et al.* (1984) who separated two major proteins of molecular masses 30 and 42 kDa in the OM-enriched fraction. Knights *et al.* (1990) proposed that polypeptide bands of molecular weights 4, 7, 9, 15, 16, 22, 23, 30, 33, 41,42, 43 and 44 kDa appeared to be located in the outer membrane.

The rough type of LPS based on SDS-PAGE profiles of biotype A strains in this study confirm the findings of Adlam (1989) and are of LPS type 3 (Ali *et al.*, 1992). According to Ali *et al.* (1992) LPS type 1 and type 2 were both smooth-type LPS with apparently identical O-antigen side-chains but with different core oligosaccharide region. LPS type 3 was rough, but not possessing O-antigen side chain, but its core-oligosaccharide region was similar, although not identical to that of type 1 LPS. However, Davies *et al.* (1992) demonstrated that most of the serotype A1 isolates that he examined possesed a high molecular mass ladder pattern in silver-stained SDS-PAGE gels characteristic of smooth-type LPS. These were designated as type 1. It has been suggested that the resulting LPS profiles were affected by the staining and extraction procedure of the LPS.

However, he demonstrated that the majority of serotype A2 isolates possesed LPS of type 3 whereas the remaining A2 isolates had a unique rough LPS type; types 4 and 5. The authors also suggested that rough type LPS types, and type 3 LPS in particular, are primarily associated with acute pneumonia in sheep whereas smooth LPS of type 1 is associated with acute pneumonia in cattle. This findings appeared to agree with our results since majority of the isolates examined were of rough type 3 LPS and they were obtained from cases of sheep and goat pneumonia.

The epidemiological significance of OMP and LPS migration patterns obtained by SDS-PAGE was summarised above and more fully discussed elsewhere (Davies *et al.*, 1994). This is the first study comparing variation in the OMP and LPS patterns of selected *P. haemolytica* strains common to two countries and may be useful in epidemiological studies.

However, using PAGE alone, any assumption that the isolates are different would be erroneous. Attempts to correlate polypeptide migration patterns with antigenic variation among pathogenic organisms by using blots with limited range of antisera showed that strains with identical polypeptide patterns can react differently (Harris *et al.*, 1979). While PAGE is an extremely powerful epidemiological tool, it is limited by inability to predict antigenicity, crucial in determining vaccine strategy.

CHAPTER 5.0 ASSESSMENT OF EFFICACY OF A NEW *P. HAEMOLYTICA* IRON-REGULATED PROTEINS (Ovipast-9-IRP^R, HOECHST, UK) VACCINE IN MALAYSIAN SHEEP FLOCKS

5.1 Evaluation of Ovipast-9-IRP^R vaccine against natural pneumonic challenge in sheep

5.1.1 Introduction

The results of epidemiological studies on the prevalence of *P. haemolytica* in Malaysia and the UK, described in Chapter 4.0 showed no real difference between the strains suggesting that vaccines containing the British strains can be used in trials in Malysian sheep flocks. Work at MRI, has shown that IRPs produced by *P. haemolytica* cells when growing in infected hosts stimulate a strong antibody response (Donachie *et al.*, 1988). These IRPs can be induced to grow *in vitro* by removal of iron from the media and their inclusion in vaccines in sheep enhances the protective efficacy of these vaccines against *P. haemolytica* challenge (Gilmour *et al.*, 1991).

Pasteurellosis of sheep is reportedly best prevented by multivalent vaccines, since there is no cross-protection is not evident between serotypes (Gilmour *et al.*, 1979, 1983). However, the efficacy of these vaccines is difficult to evaluate without recourse to experimental challenge (Sharp *et al.*, 1978). Existing serological methods have not proved suitable for detecting protective serum antibody responses to individual serotypes. The IHA test is highly specific for capsule polysaccharide antigen, but is insufficiently sensitive, while ELISA based on SSE extract antigen (capsule polysaccharide, protein, LPS) detects cross-reacting antibodies (Burrells *et al.*, 1979).

Various *pasteurella* antigens have been employed in ELISA including SSE (Burrells *et al.*, 1979), phenol water extract (Donachie *et al.*, 1984a), saline extract (Confer *et al.*, 1985), LPS (Confer *et al.*, 1986) formalised cells (Opuda-Asibo *et al.*, 1986) and cytotoxin (Mosier *et al.*, 1986). These preparations were used to measure antibodies against experimental single serotype vaccines. Cross-reactions between serotypes occur with crude antigens such as SSE (Donachie *et al.*, 1983) and with more defined preparation such as phenol water extract and LPS (Donachie *et al.*, 1984b, Confer *et al.*, 1986).

This study was conducted to determine the relative value of the new vaccine containing iron-regulated proteins (Ovipast-9-IRP^{R}, Hoechst, UK) in the control of pasteurellosis under natural conditions using serological monitoring. IHA test and ELISAs were used to quantify antibody responses against relevant specific antigens. The

incidence of disease and the serological changes in these treated groups were compared with those in control untreated groups of animals from the same farms over the same periods.

5.1.2 Materials and Methods

Vaccines study design: For the purpose of this study, arrangements were made with a number of sheep farms of various flock sizes, whereby 1000 animals could be suitably treated and identified. Four farms were choosen for detailed field study based on their willingness to co-operate and their close location to MARDI. Farm A and Farm B were medium sized farms with 500-2000 heads of animals. They were situated at MARDI stations at Serdang and Kluang. Farm C belong to a private company, Kumpulan Guthrie which has a total sheep population of 10,000 heads raised under oil palm plantations. Farm D belong to a smallholder farmer who owned 200 heads of sheep (see Appendix 1). The flock comprised of local sheep (Malin) and cross-bred (Dorset-Malin) and normally were allowed to graze under oil palm or rubber areas between 7 am to 4 pm. They were housed during the night in platform houses made of wood.

These animals were to receive no special care and were to be treated in the same manner as all other animals in the farms. The study commenced in November 1992 and ended in October, 1993. During this period, 750 animals were vaccinated with Ovipast--9-IRP^{R} vaccine and 250 animals were identified as control unvaccinated

animals. The ewes, their offspring and the rams were included in this study. The farm managers identified each animal at birth with an ear tag and maintained lambing and individual treatment records for the occurence of pneumonia.

At the start of the study, a serological profile of the flock was establised by testing several groups of sheep including ewes, rams, weaning lambs and growers of various ages.

The vaccines were administered in the dosage recommended by the manufacturer (2 ml/animal given subcutaneously). Animals in the vaccinated groups received two vaccinations 28 days apart.

Immediately prior to vaccination, blood samples were collected from 20 per cent of the trial groups. In some of the farms the blood samples were collected weekly for 8 weeks, but in others only 5 collections were made, at days 0, 1 week, 3 5 weeks and 7 weeks post-vaccination. All sheep that died were sent to the regional diagnostic laboratory for autopsies.

Serological Methods: Sera were analysed for IHA antibody and for specific antibody to 35 kDa IRP antigen and anti-A1 capsule using ELISA, as described in the general Materials and Methods.

5.1.3 Results

The mean serum antibody responses to each of these antigens of the vaccinated and control groups plotted against times for the farm A, farm B, farm C and farm D are shown in Fig. 5.1a-5.1d, Fig. 5.2a-5.2d, Fig. 5.3a-5.3d and Fig. 5.4a-5.4d respectively. There were significant differences ($p<0.05$) in the antibody responses between vaccinates and controls at various post-vaccination time intervals for IHA antibody, 35kDa and anti-A1 capsule antigens of *P. haemolytica* in all the trial farms.

Prevalence of IHA antibody in the flock: At the start of the study, the prevalence of the IHA antibodies in the vaccinated flock in all four sheep farms was not significantly diffferent ($p>0.05$) compared with control unvaccinated flocks (Fig. 5.1a-5.1d). The mean IHA antibody titres of the vaccinated animals in all the farms significantly increased shortly after administration of the IRP vaccines from mean titres of 1:4 at the start of the study to 1:64 one week after vaccination. There was no significant change in the mean titres of IHA antibody in the control animals throughout the study period.

One week after adminitration of the vaccines there were significant differences ($P<0.05$) in mean IHA antibody titres between vaccinated and unvaccinated groups. The vaccinated group's mean antibody titre was significantly greater than the mean titre of the unvaccinated control at weeks 1, 3 and 5. The mean pre-vaccination

IHA titres at days 0 were low (1:4). After the first injection the mean titre of the animals had increased by day 7 to 1:64, then decreased until day 21 and increased significantly after booster to level >1:128 and thereafter. The mean titre of the unvaccinated control remained low from days 0 to 35.

The presence of 35 kDa antibody: There were significant differences in 35 kDa IRP antigen mean optical density readings (OD_{492}) between vaccinated and unvaccinated groups in all the farms. The mean OD readings for the vaccinated group was significantly greater than the mean OD for the unvaccinated group at 1 week, 3 weeks, 5 weeks and 7 weeks post-vaccinations ($p<0.05$). The mean OD readings of the vaccinated group increased from OD 0.45 on day 0 to OD 0.98 on day 7, but decreased sharply thereafter and increased again after a second vaccination on day 35 and remained unchanged thereafter. Its OD readings reached maximum 7 days after second injection. The sera from the unvaccinated control group on day 0 showed mean OD readings of 0.4 because more than half of the animals had high passive antibody levels. This groups mean OD decreased to OD of 0.3 on day 35.

The presence of anti-A1 capsular antibody: There were significant differences in anti-A1 capsular mean OD readings ($P<0.05$) between vaccinated and non-vaccinated groups in all the farms. The mean OD for the vaccinated group was significantly greater than the mean OD for the unvaccinated group on weeks 1, 3 and 5. The mean OD readings on days 7 was 1.1. The mean OD reached a maximum 7 days

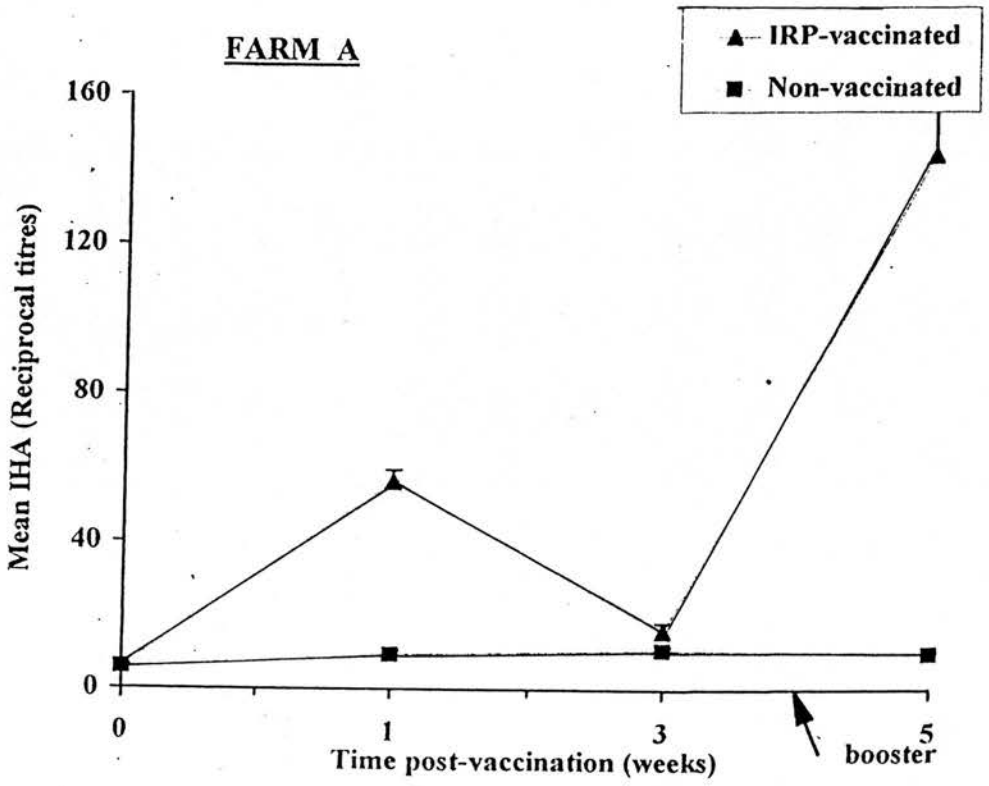

Fig. 5.1a. Mean IHA antibody response to *P. haemolytica* antigens in sheep vaccinated with Ovipast-9-IRP vaccine

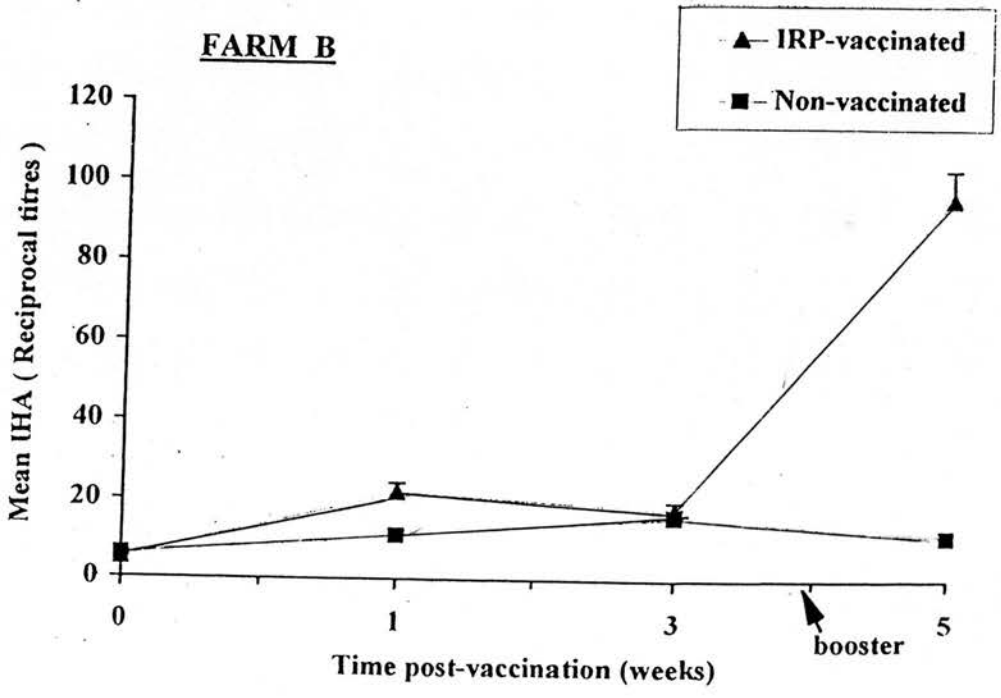

Fig. 5.1b. Mean IHA antibody response to *P. haemolytica* antigens in sheep vaccinated with Ovipast-9-IRP vaccine

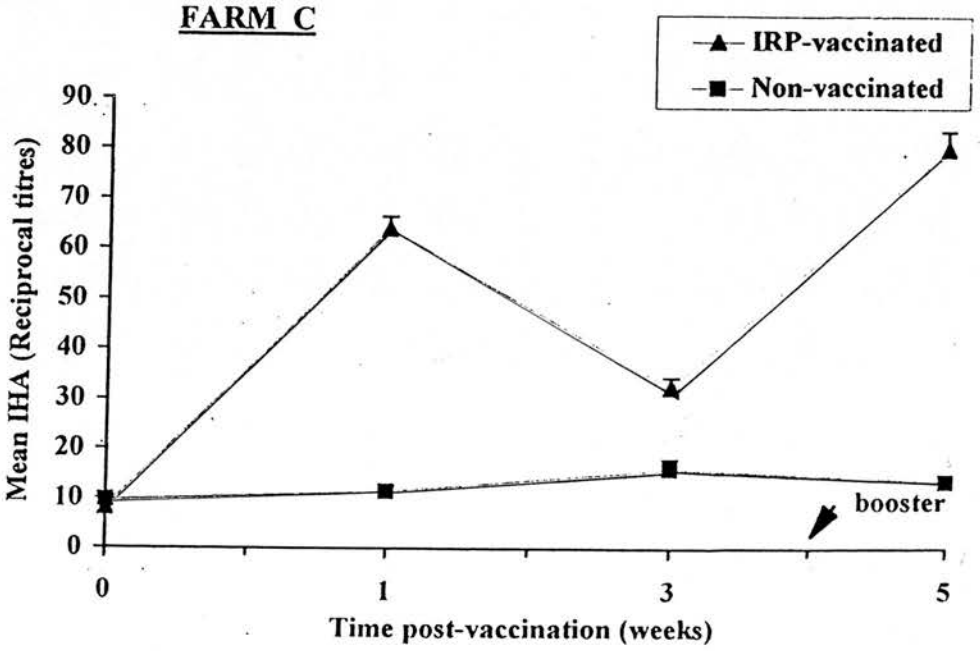

Fig. 5.1c. Mean IHA antibody response to *P. haemolytica* antigens in sheep vaccinated with Ovipast-9-IRP vaccine

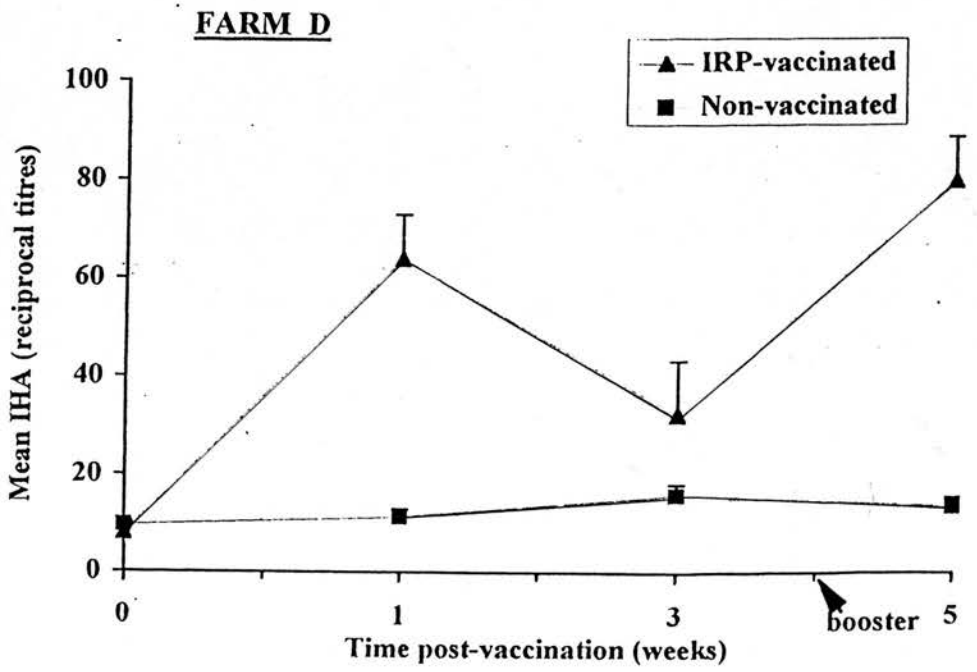

Fig. 5.1d. Mean IHA antibody response to *P. haemolytica* antigens in sheep vaccinated with Ovipast-9-IRP vaccine

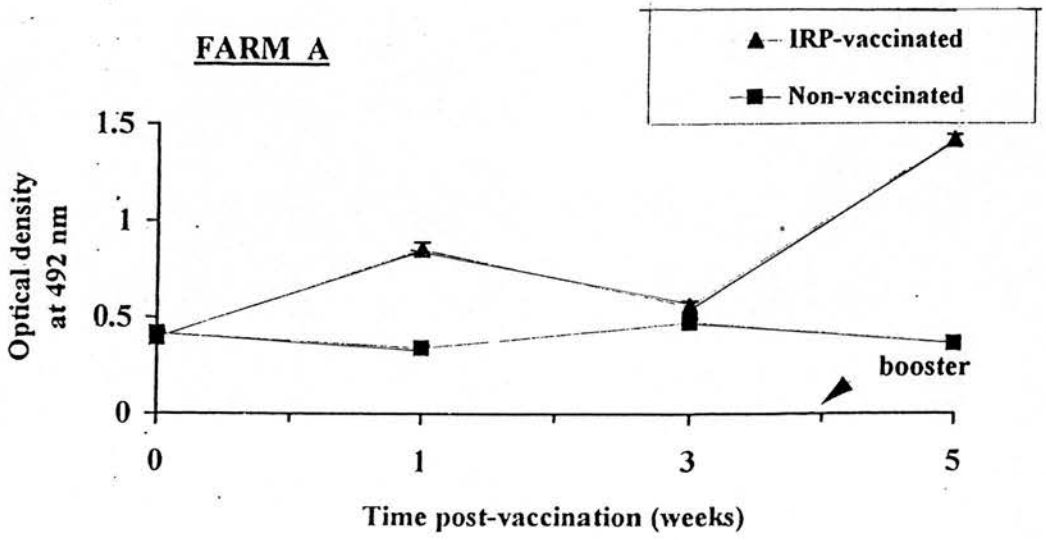

Fig. 5.2a. Antibody response to *P. haemolytica* A2 35K IRP in sheep vaccinated with Ovipast-9-IRP^R vaccine

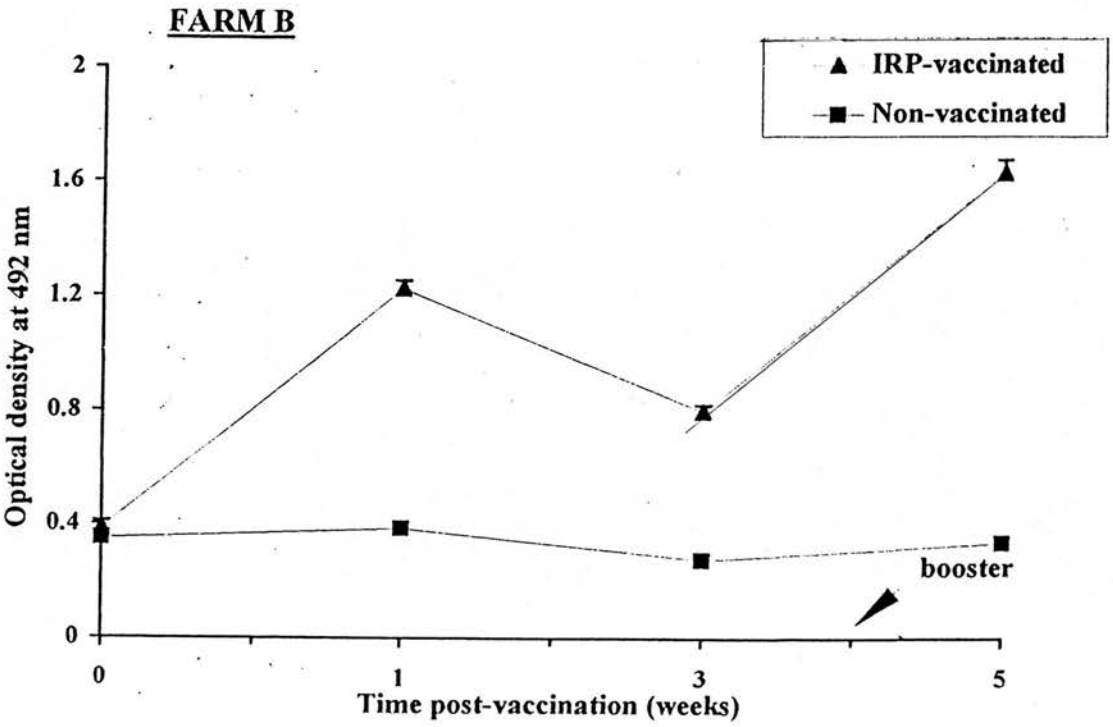

Fig. 5.2b. Antibody response to *P. haemolytica* A2 35K in sheep vaccinated with Ovipast-9-IRP^R vaccine

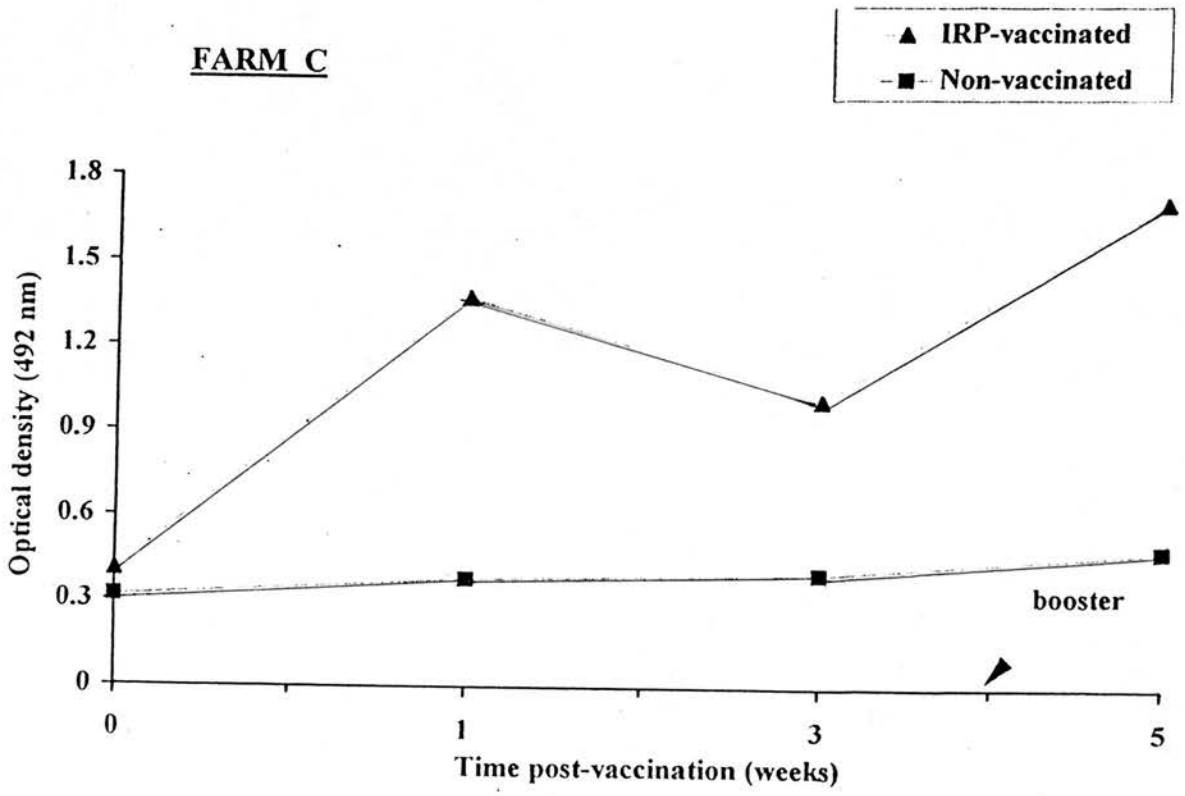

Fig. 5.2c. Antibody response to *P. haemolytica* A2 35K IRP in sheep vaccinated with Ovipast-9-IRP vaccine

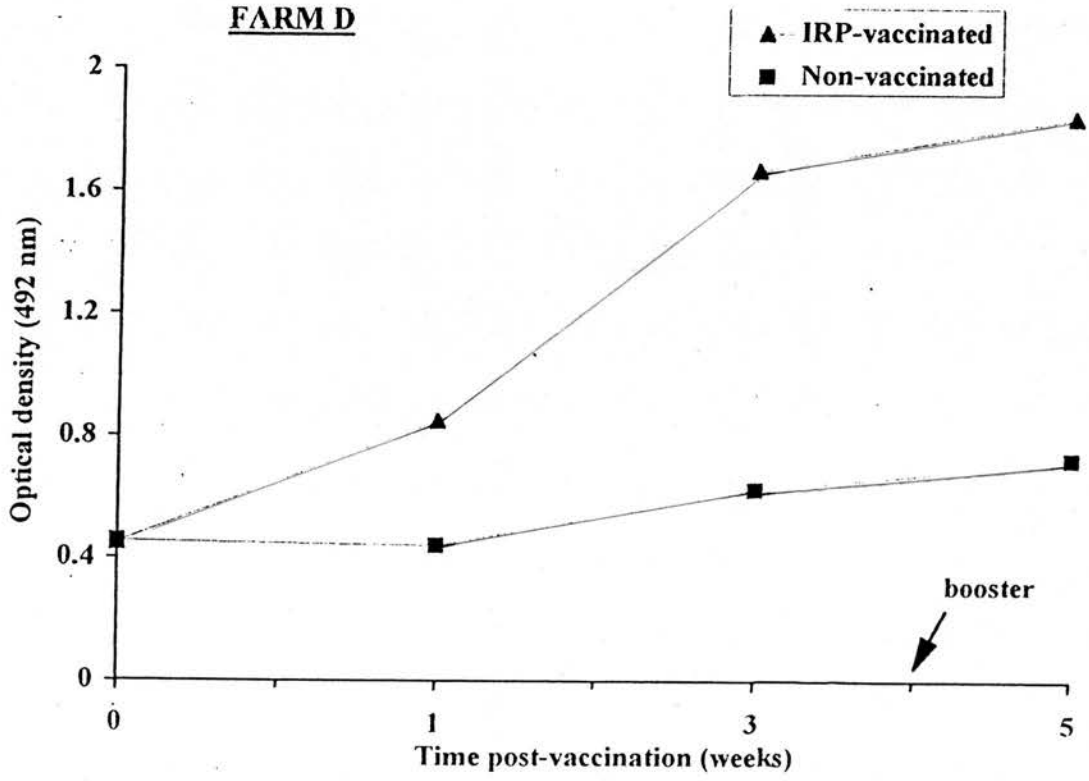

Fig. 5.2d. Antibody response to *P. haemolytica* A2 35K IRP in sheep vaccinated with Ovipast-9-IRP vaccine

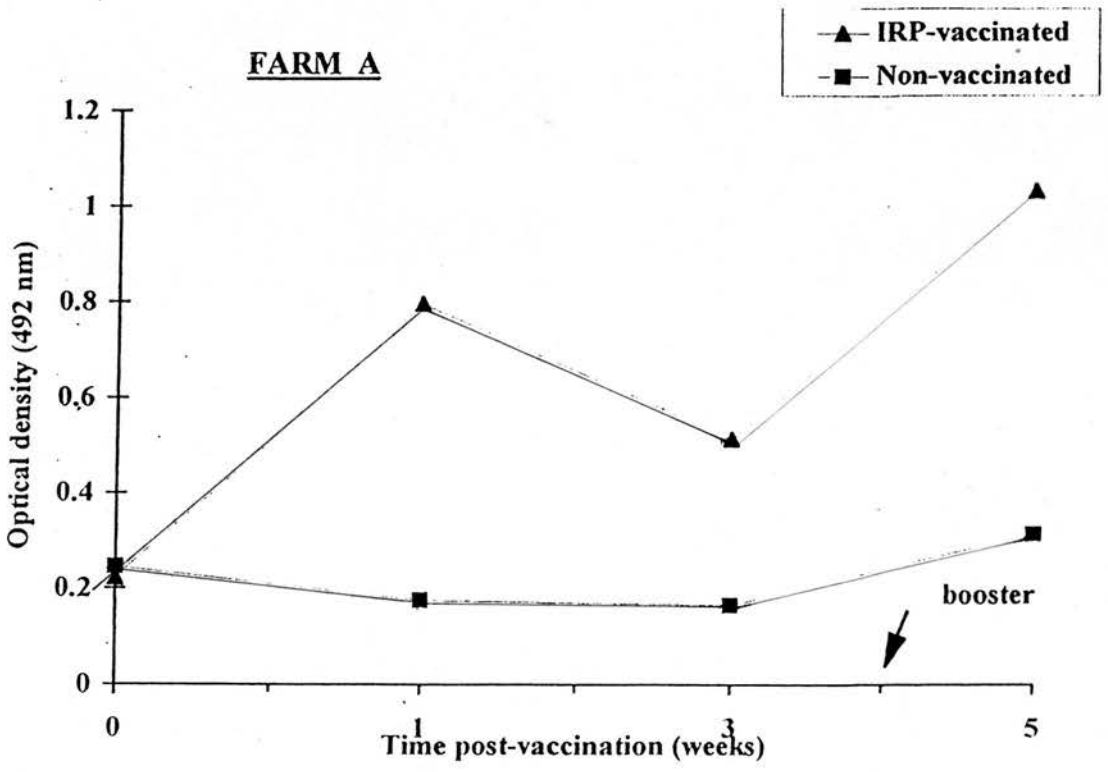

Fig. 5.3a. Antibody response to *P. haemolytica* anti-A1 capsular antigen in sheep vaccinated with Ovipast-9-IRP vaccine

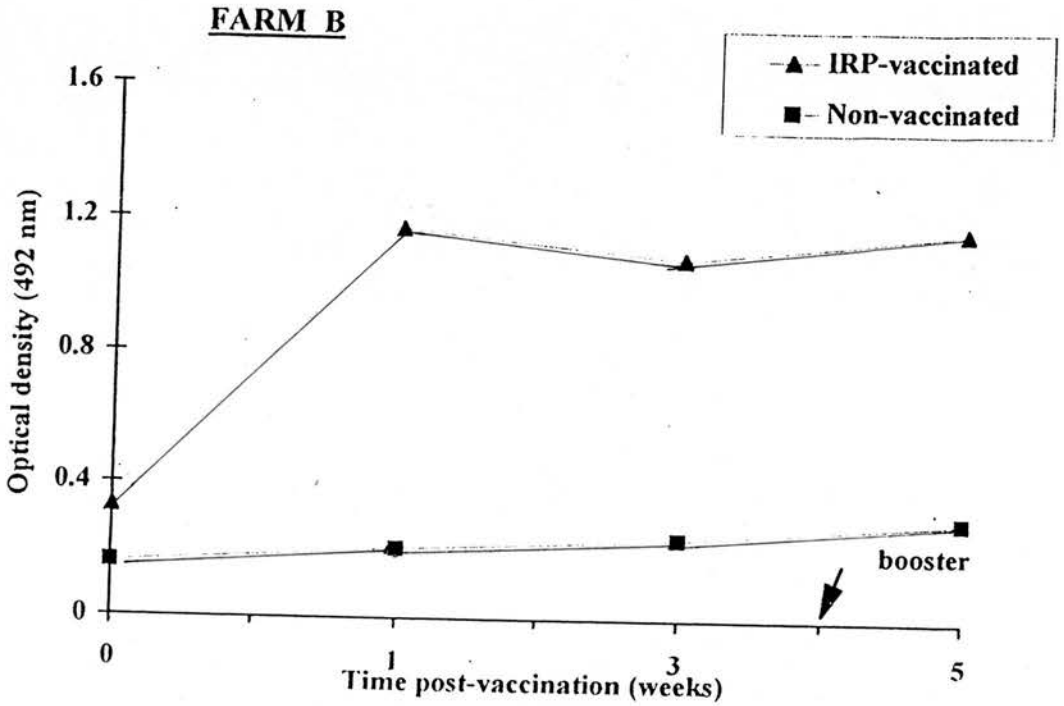

Fig. 5.3b. Antibody response to *P. haemolytica* anti-A1 capsular antigen in sheep vaccinated with Ovipast-9-IRP vaccine

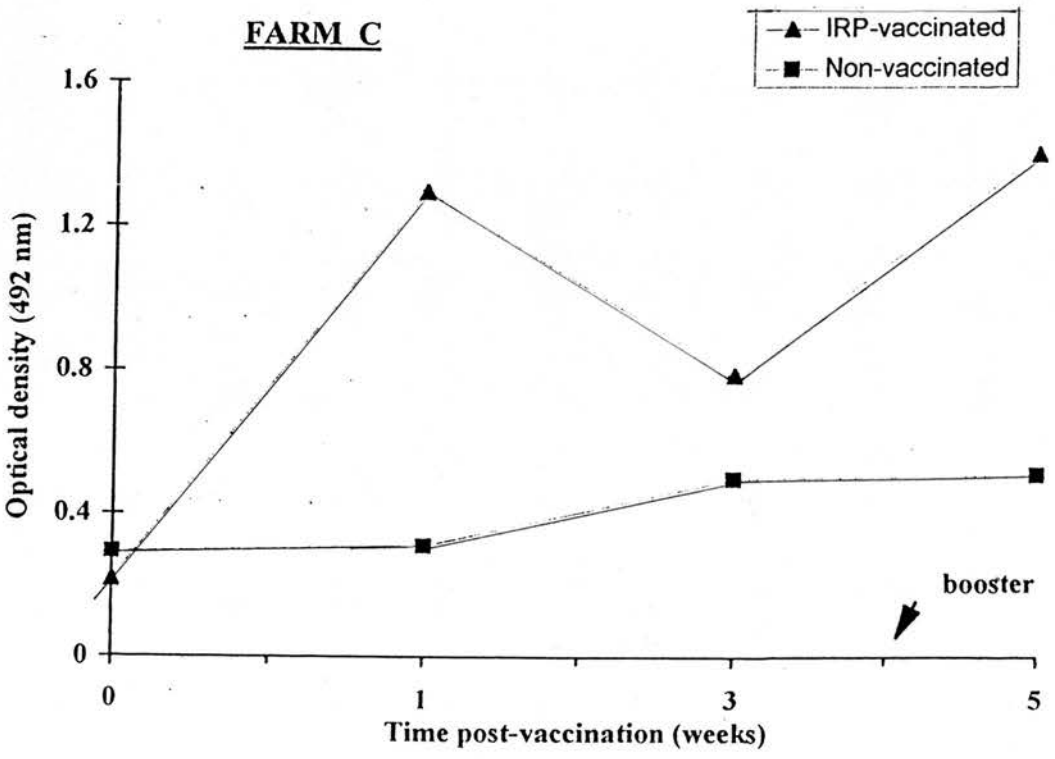

Fig. 5.3c. Antibody response to *P. haemolytica* anti-A1 capsular antigen in sheep vaccinated with Ovipast-9-IRP vaccine

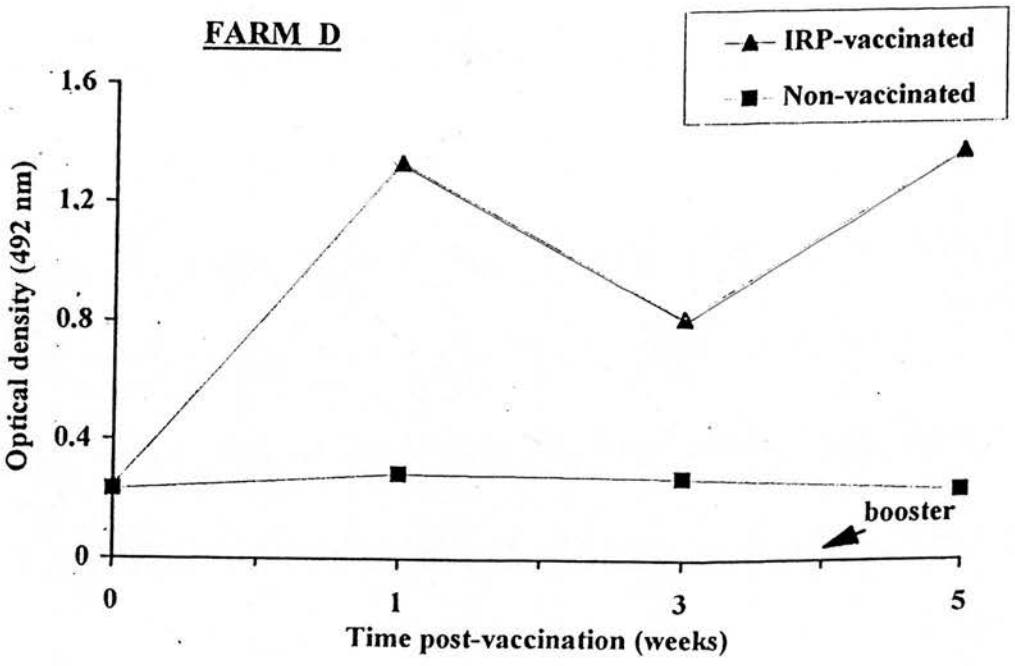

Fig. 5.3d. Antibody response to *P. haemolytica* anti-A1 antigen in sheep vaccinated with Ovipast-9-IRP vaccine

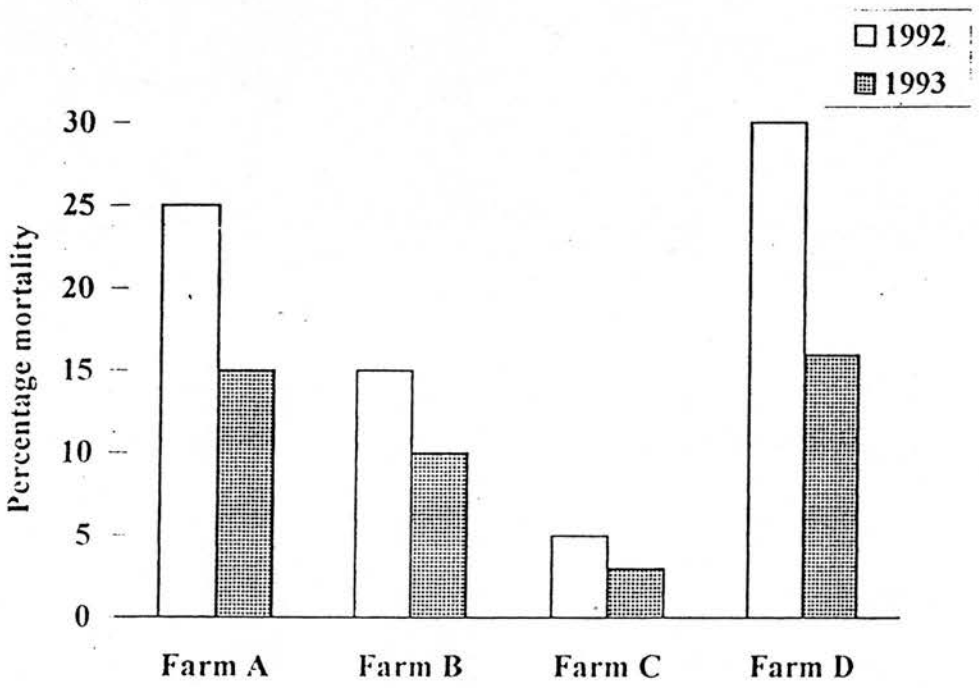

Fig. 5.5. Death due to pneumonic pasteurellosis in the IRP vaccine trial sheep farms in the year 1992-1993.

after the second injection. The mean OD of the unvaccinated control was 0.2 on day 0 and changed little until week 5.

In all the vaccinated farms the production performance in terms of animal survivality during the outbreaks of pasteurellosis during the trial period (1992-1993) was improved as compared to non-vaccinated farms. Although these mortality figures were not significantly different ($P>0.05$), the vaccinated farms recorded reduced ewe and lamb mortality. Unvaccinated flocks had 10%, 6%, 3% and 12% higher total annual sheep mortality due to pasteurellosis in farm A, farm B, farm C and farm D respectively compared to vaccinated flocks (Figure 5.5).

5.1.4 Discussion

In this study the magnitude of antibody response against 35 kDa IRP antigen, anti-A1 capsule and IHA antibody in conventional sheep vaccinated with a novel vaccine containing iron-reguaited proteins (Ovipast-9-IRP^R, Hoechst, UK) was measured. A high level of antibody response against these antigens was demonstrated as has previously been reported by other investigators (Donachie *et al.*,1988, Gilmour *et al.*, 1991).

As it is not possible to produce pneumonic pasteurellosis regularly in conventionally reared sheep by exposure to aerosols of *P. haemolytica* or intratracheal

inoculation, the assessment of vaccine efficacy was based on the measurement of the serum antibody titres. There were wide variations in the serum IHA antibody titres. In vaccinated animals at the start of the trial the serum antibody titres ranged from 8 to 128 and in unvaccinated controls ranged from 4 to 128. The considerable differences in the mean titres between groups of vaccinated and unvaccinated sheep were not significantly significant ($p>0.05$) because of these wide ranges of values. However, after vaccination, the IHA titres of the vaccinated animals increased significantly compared to unvaccinated counterparts resulting in significant values ($p<0.05$). The pattern of response to 35 kDa IRP antigen and anti-A1 capsule was similar to the IHA responses.

The results of the serological studies on farms A, B, C and D at the commencement of study were very similar. A high percentage of the sheep had detectable antibodies against 35 kDa IRP and high antibody levels to A1 capsule antigen and IHA titres. Higher OD readings in the sera of the animals at the start of the experiment are to be expected because of earlier exposure of the conventional animals to the infection of *P. haemolytica.*

In farm C, higher ELISA and IHA antibody levels were observed in many lambs. This was probably due to maternal antibodies in the colostrum of the positive ewes. The results were to be expected because strict vaccination against pasteurellosis was practised in the farm. The titre of these passive antibodies rapidly decreased to reach a minimum three months later. However large number of lambs in farm D, where

vaccination programmes were also included in their husbandary practices, had much lower ELISA antibody levels and IHA titres when compared to the other farms and some of the lambs had negative titres. The explanation for this situation could possibly be due to improper handling of the vaccines or the animals were not vaccinated as recommended.

Serological monitoring studies indicate that the vaccine stimulated a good response in vaccinates although a high level of non-specific reactivity was observed in a number of animals in the control and vaccinated group. Sera from vaccinated and non-vaccinated sheep were tested by ELISA for 35 kDa IRP antibodies, capsular A1 antibodies and IHA titres. All vaccinated animals had high ELISA antibody levels and high IHA titres after first vaccination. The antibody levels increased rapidly after booster, but declined sharply after that. Peak immune responses were observed 1 week after first vaccination, but then declined rapidly in most animals when tested for IRP antibodies. In sheep which were vaccinated twice, the titres clearly increased after the second vaccination. Control animals had low antibodies levels to all the antigens tested.

Although there is some doubt as to the protective value of serum alone (Wells *et al.*, 1979) and therefore of the significance of serum antibody titres, the titres reported in this trials are of the same order as those obtained in SPF lambs given the same vaccine to lambs and shown to have been protected on challenge (Gilmour *et al.*, 1991).

The study indicates that vaccination alone with vaccine containing IRP results in high immunogenicity and high protection of sheep under field condition. It appeared that the IRPs vacccine stimulated the sheep immune system to elicit antibody response to protect sheep against clinical pasteurellosis.

However, six months after the vaccination trials, the serum antibody levels of ewes tested on farms A and B were lower than at the beginning of the study, some of them even below the positive threshold. Therefore the duration and quality of the immunity conferred by the vaccination warrants further examination. Further evaluation has to be carried out to permit confirmation of the effectiveness of the dosage of the vaccine, the vaccine schedule and the timing of the booster dose.

5.2 Evaluation of a new *P. haemolytica* IRP vaccine (Ovipast-9-IRP^R, Hoechst, UK) against experimental *P. haemolytica* A2 challenge in conventional sheep

5.2.1 Introduction

Development of improved *P. haemolytica* vaccines for sheep and goats has progressed markedly since it has been possible to reproduce pneumonic pasteurellosis experimentally (Gilmour *et al.*, 1983). However, a major limitation has been the necessity to rear lambs in specific pathogen free (SPF) conditions for such experiments. Although conventional animals may have pre-existing experience of *P. haemolytica* infection, it was considered worthwhile to attempt vaccination-challenge experiments in conventionally reared lambs.

This study was conducted to test the efficacy of a novel *P. haemolytica* vaccine containing iron regulated proteins (Ovipast-9-IRP^R, Hoechst, UK) against experimental *P. haemolytica* A2 challenge.

5.2.2 Materials and Methods

Animals and experimental design: These were lambs from a mixed breed of Dorset-Malin sheep. The lambs were randomly allocated to vaccinated and unvaccinated control groups and were housed in isolation pens in groups of ten with

equal numbers of vaccinates and controls. They were fed with cut grass and water *ad lib* and concentrates given at 200 g/head daily.

Experimental design

	Vaccination *	Vaccination	Challenge
Group(n=10)			
Vaccinated	7	35	47
Unvaccinated	-	-	47

***- days after commencement of trials**

Vaccines and challenge infection: The lambs were vaccinated subcutaneously (2 ml dose) with two doses of Ovipast-9-IRP^{R} vaccines with an interval of 28 days between injections. The animals were bled for serology by jugular venipuncture (10 ml vacutainers) before vaccination at days 0 and weekly for 6 weeks. The lambs were subjected to transportation stress followed by three consesecutive injections of 10 mg per lamb of dexamethasone (Dexasone^{R}, Atlantis, Germany) and after the last dexamethasone injection the animals were immediately infected intra-tracheally with inoculation of 6 ml suspension of *P. haemolytica* A2 (2 x 10^{9} cfu /ml).

Clinical and pathological assessment: The clinical appearance of each

lamb was monitored for 72 h after infection. Any lambs which died were examined post mortem. Samples were taken for bacteriological examination. Clinical and pathological findings were scored by methods described by Gilmour *et al.*, (1983). The daily clinical score for each lamb was calculated on the basis points alloted to dullness, respiratory abnormality and fever. The lambs that survived after 3 days post-challenge were euthanised with 10 ml of saturated magnesium sulphate per animal intravenously. Necropsies were carried out immediately and samples sent for bacteriological and pathological examinations.

Serological Methods: Sera were analysed for IHA antibody and for specific antibody to 35 kDa IRP antigen and anti-A1 capsule using ELISA, as described in the general Materials and Methods.

5.2.3 Results

The group mean clinical, lung, bacteriological and total disease scores are shown in Table 5.3a.

Clinical observations: Six hours after exposure to *P. haemolytica* A2 challenge two lambs in the control group were severely affected while three others were dull and anorexic. Two lambs in the control group died 24 h later. Febrile condition were observed in five of the ten vaccinated lambs but all the lambs survived,

though one was dull, but still standing. No other clinical abnormality were observed.

At necropsy, the lung lesions characteristic of pasteurellosis were noted in 3 of the unvaccinated animals. Profuse pure growth of challenge organisms were isolated from those lesions. The histologic appearance of the lesions was that of a supparative bronchopneumonia with alveolar collapse. There was infiltration of polymorphs in the bronchioles and serum leakage and several multinucleate giant cells were present. Findings were consistent with *P. haemolytica* pneumonia..

No specific lesions were visible in the lungs of the IRP vaccinates, but acute congestion and petechiation over the heart were visible in 3 of the animals. *P. haemolytica* was recovered in two of the lungs.

The IRP vaccinates had mean total disease scores that were 2.7 times lower than those for unvaccinated animals but not significantly different ($p>0.05$) (Table 5.3a).

Table 5.3a. Group mean clinical, bacteriology, lung lesion and total disease scores for sheep vaccinated and unvaccinated with Ovipast-9-IRP^R vaccine.

Vaccine	Clinical Score (± SE)	Lung Lesion Score (± SE)	Bacteriology Score (± SE)	Total Disease Score (± SE)
Ovipast-9-IRP^R vaccine	1.8±0.66	1.0±0.67	0.8±0.53	3.6±1.5
Unvaccinated (control)	5.4±1.7	3.0±1.5	2.4±1.2	9.6±4.1

Serological evaluation: The results of the serum antibody responses to 35 kDa IRP of A2 and anti-capsualr antigens measured by ELISA are shown in Table 5.3b. The Figures 5.4 - 5.6 showed the serum antibody responses to *P. haemolytica* A2 35 kDa IRP and anti-A1 capsule antigens and IHA response plotted against time post-vaccinations.

Table 5.3b. Serum IHA titres and antibody response to 35 kDa IRP of *P. haemolytica* A2 and anti-A1 capsular antigens measured by ELISA in sheep vaccinated with Ovipast-9-IRP^R vaccine.

Vaccine	Mean antibody response(±SE)				MeanIHA(titre^{-1})	
	35 kDa IRP		anti-A1 capsule		Pre-vacc.	Post-vacc.
	Pre-vacc.	Post.-vacc	Pre-vacc.	Post-vacc.		
Ovipast-9-IRP^R vaccine	0.12±0.001	0.29±0.021	0.71±0.035	1.37±0.03	9.6±1.5	166.4±26
Unvaccinated (control)	0.11±0.005	0.19±0.04	0.4±0.032	0.38±0.03	6.4±1.2	15.6±3.1

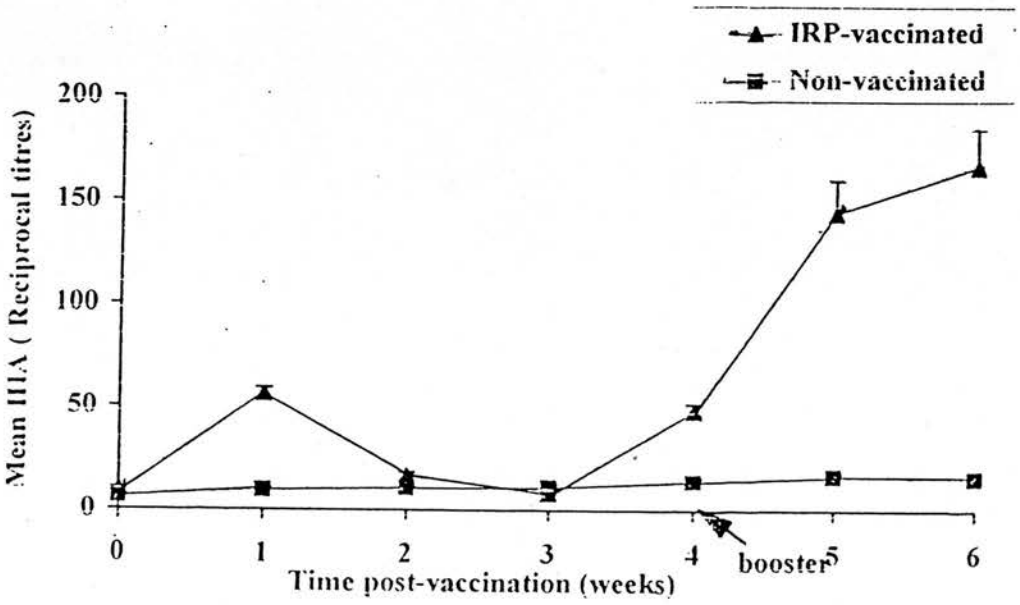

Fig. 5.4. Mean IHA antibody response to *P. haemolytica* A2 antigens in sheep vaccinated with Ovipast-9-IRP^{R} vaccine

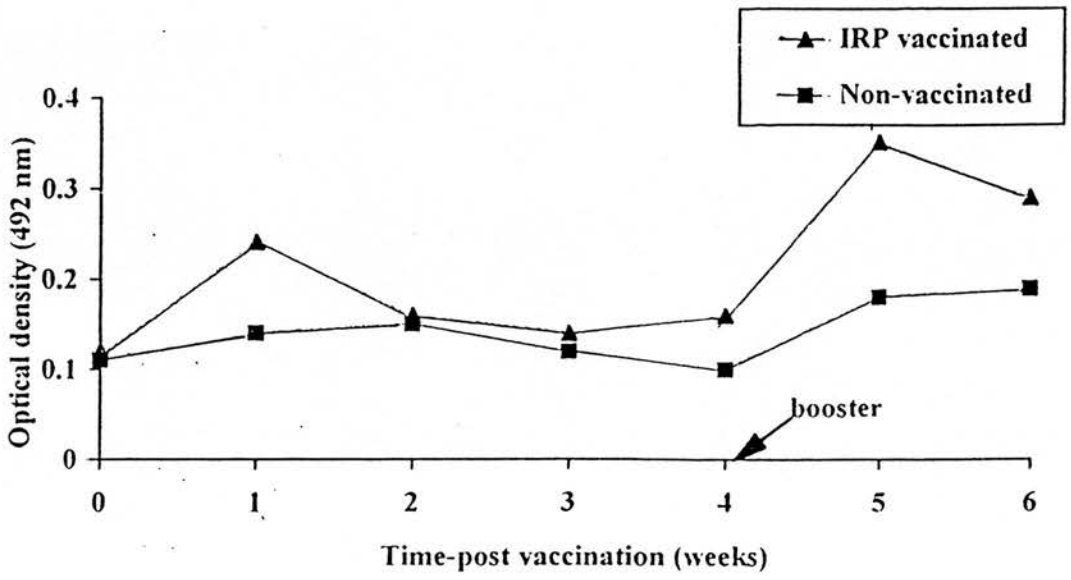

Figure 5.5. Antibody response to *P. haemolytica* A2 35 kDa IRP in sheep vaccinated with Ovipast-9-IRP^R vaccine

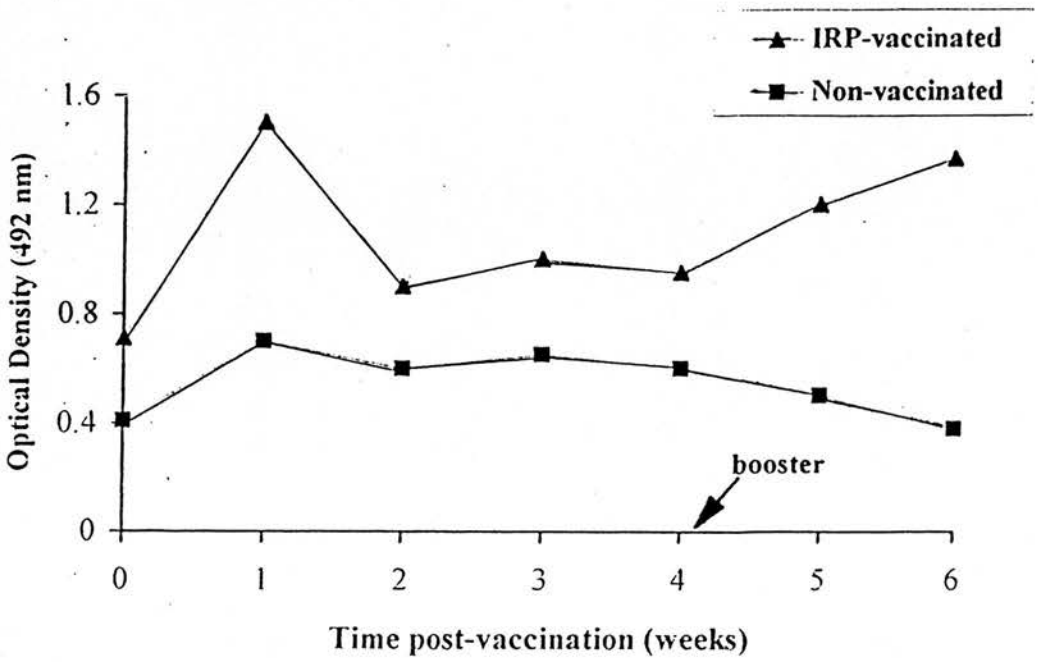

Fig. 5.6. Antibody response to *P. haemolytica* anti-A1 capsular antigen in sheep vaccinated with Ovipast-9-IRP^R vaccine

Serology indicated that the vaccine stimulated a good response in vaccinates, although a high level of non-specific reactivity was observed in the both groups.

Sera from vaccinated and non-vaccinated sheep were tested by ELISA for 35 kDa IRP and anti-A1 capsular A1 antibodies. There were significant differences ($P<0.05$) in the mean antibody titres against the antigens tested between vaccinated and unvaccinated groups. There was also a rise in IHA titres in the vaccinated group but due to the wide range of titres recorded (between 1:4 to 1:256) the differences between the groups were not statistically significant ($p>0.05$)

5.2.4 Discussion

Although there were no significant differences ($P>0.05$) in the total disease scores between vaccinated and the unvaccinated controls animals, more lambs died in the control group. However, vaccinated animals had higher mean antibody titres against the antigens tested and it appeared that high titres were correlated with protection against clinical pasteurellosis. The results are in agreement with that reported by Gilmour *et al.* (1991) who demonstrated that *P. haemolytica* A2 extract vaccine containing IRPs protected SPF lambs. Previously, protection against disease caused by A2 serotype has been demonstrated in SPF lambs recovered from experimental infection (Donachie *et al.*, 1986) or passively protected with immune serum (Jones *et al.*, 1989).

Antibody response was elicited in vaccinated animals against 35 kDa IRP antigen and anti-A1 capsule. High IHA titre was also elicited in the vaccinated group. However, a few control unvaccinated lambs also showed strong antibody responses. This may due to cross reactivity with other other bacterial species since *P. haemolytica* is not present in any of the lambs prior to challenge.

The results from this study demonstrated that there was some indication of protection afforded by Ovipast 9 IRP^R vaccine in the vaccinated conventional animals. However, the reproducibility of the results may be poor due to the nature of on non-specific immune reactions in conventional animals as opposed to SPF animals. More challenge exposure studies using conventional animals are suggested for the future to evaluate the degree of protection conferred by this vaccine against pneumonic pasteurellosis.

CHAPTER 6.0 PRODUCTION OF *P. HAEMOLYTICA* SEROTYPE A2 POLYSACCHARIDE CAPSULE

The antigens responsible for conferring protection against certain *P. haemolytica* serotyypes A are known to be contained in extracts prepared by sodium salicylate, saline and phenol treatment from these cells (Gilmour *et al.*, 1979 and 1983). These extracts are presumed to contain surface components and they include the serotype specific antigen as sheep vaccinated with this extract in adjuvant produce high levels of antibody against the serotype antigen (Wells *et al.*, 1979).

Identification of the specific antigen or antigens involved in protection would help in understanding the immunology of the disease. Adlam *et al.* (1987) have shown that capsular polysaccharide from culture supernatant of *P. haemolytica* serotype A2, the most common serotype isolated from pasteurellosis cases in sheep, has a capsular structure identical to the capsular polysaccharide (also known as colominic acid) of *N. meningitidis* group B and *E. coli* K1; an α(2→8)-linked heteropolymer of N-acetyl neuraminic acid.

The conditions governing the production of colominic acid and dextran (α-1,4-linked dextran) polymers of *P. haemolytica* A2 organisms remain to be evaluated. The aim of these series of experiments were to determine the optimum production conditions for A2 capsule polysaccharide.

6.1 Extraction of capsular material from *P. haemolytica* serotype A2

6.1.1 Introduction

A soluble capsular material was demonstrated when *P. haemolytica* was cultured overnight on a fresh beef infusion agar plate (Carter, 1956). The author stated that this substance was polysaccharide in nature and removed it from organisms by heating a culture of *P. haemolytica* at 56° C for 30 min in water or normal saline solution. *P. haemolytica* cultures produced capsular material early (4 to 6 h) in their growth cycle as demonstrated by the direct flourescent antibody (FA) technique, using antiserum to whole organism (Gentry *et al.*, 1981). By this technique the author further showed that the capsule was found to dissappear gradually as the culture aged so that little capsule material remained on the cells after approximately 16 to 22 h of incubation.

The purpose of this study was to estimate the yield of this capsule

produced from the different growth phase of *P. haemolytica* A2 in different types of culture media.

6.1.2 Materials and methods

P. haemolytica A2 (strain EO-200) cells were grown in three different types of media, viz. nutrient broth, brain heart infusion and trypticase soya broth. They were cultured at different incubation times as shown in Table 6.1.

6.1.3 Results

The production of capsular materials in the various culture media at different duration of culture is shown in Table 6.1. Sialic acid determination indicates the amount of colominic acid produced.

There were no significant differences in terms of sialic acid and protein content composition between the media tested ($P>0.05$). The production of sialic acid and protein was highest in the 6 h culture in nutrient broth, but are not significantly different when compared to the 18 h or 24 h culture ($p>0.05$). The nutrient broth (Gibco, No. 2) at 6 h culture supported 6×10^8 cfu/ml viable counts of *P. haemolytica* A2 and contained 1.7 mg/ml protein and 0.06 mg/ml sialic acid respectively.

Table 6.1 Protein and Sialic acid determination on the *P. haemolytica* A2 capsular extracts[a]

Culture Time	Parameter	Types of Culture Media		
		Nutrient Broth	Brain Heart Infusion	Tryptic Soya Broth
6 h	V	6.5×10^8	4.8×10^7	5.1×10^6
	P	1.7	0.8	0.7
	S	0.06	0.04	0.06
18 h	V	6.0×10^8	4.1×10^7	4.0×10^6
	P	1.25	0.8	0.85
	S	0.06	0.05	0.04
24 h	V	4.1×10^5	3.1×10^4	4.0×10^4
	P	1.2	1.0	0.8
	S	0.05	0.04	0.05

V - viable counts (cfu/ml)
S - average sialic acidconcentration(mg/ml)
P - average protein concentration (mg/ml)
a - average results from 4 trials

6.1.4 Discussion

The capsular extracts were shown to contain protein and sialic acid. It was not clearly understood how much of these components were leached from the cell interior

and how much were truly of capsular origin. Gentry *et al.* (1981) showed that cells from early logarithmic phase cultures of *P. haemolytica* A1 were encapsulated and the capsular material disappears gradually as the culture ages. In this study it was shown that capsular material could be maximally recovered from the early phase culture. The amount of sialic acid present was relatively constant (0.04-0.06 mg/ml) regardless of different culture media used. The protein content however varied greatly ranging from 0.7-1.7 mg/ml culture supernatant. The nutrient broth extracts contained 2-2.5 times more protein than that obtained from either BHI or TSB media and 1.5 times more sialic acid than BHI media. These differences were thought to be due to the availability of more nutritive products to support better growth of encapsulated cells of the bacteria.

From these results it can be shown that the media and the period of culture of choice for extracting maximal amount of protein and sialic acid from A2 cells was determined to be a 6 h culture nutrient broth media.

6.2 The effect of temperature on the production of capsular materials of *P. haemolytica* A2

6.2.1 Introduction

Earlier experiments have demontrated that cultures of *P. haemolytica*

produce optimal capsular materials at 6 h in their growth cycle in nutrient broth culture. Suspension media and extraction temperatures have been shown to influence the yield of the capsular materials (Gentry *et al.*, 1981). The purpose of the present study was to determine the optimal extraction temperatures for maximal production of *P. haemolytica* A2 capsular materials grown in solid and liquid culture media.

6.2.2 Materials and Methods

6.2.2.1 Extraction of capsular materials of *P. haemolytica* A2 grown on solid media (Blood Agar)

P. haemolytica A2 (strain EO-200) was cultured on twelve 9 cm. blood agar plates and incubated for 6 h. Confluent growth was carefully removed and suspended in 20 ml PBS. The bacterial suspension was heated in a water bath at different temperatures viz., at 37℃ and 41℃ followed by centrifugation at 17 000 *g* for 15 min. The supernatant was removed and sterile filtered through 0.2 μm membrane (Millipore, UK).

Viable counts were performed on the pre and post-extraction bacterial supernatant by the spot-plate method. Protein and sialic acid content were measured as described in the general Materials and Methods.

6.2.2.2 Extraction of capsular material of *P. haemolytica* A2 grown in liquid media (Nutrient Broth)

P. haemolytica A2 (Strain EO-200) was grown in 20 ml nutrient broth (Gibco No. 2) and incubated at 37°C overnight. The seed culture was inoculated in 1 litre nutrient broth. Half of the bacterial culture was incubated at 37°C and the remainder incubated at 41°C in a water bath for 6 h. The bacterial suspension were centrifuged at 17 000 *g* for 15 min and viable count, protein and sialic acid composition were measured as described in the general Materials and Methods section.

6.2.3 Results

The production of capsular materials on blood agar and in nutrient broth at extraction temperatures of 37° C and 41° C is shown in Table 6.2

The growth of *P. haemolytica* was significantly better in the solid media (blood agar) with 3.3×10^2 cfu/ml more viable counts compared to the liquid media (nutrient broth) ($P<0.05$). The growth was not affected by the temperature under study. However, when comparing the composition of the sialic acid and protein between the media it was found that there were no significant difference in sialic acid and protein content composition ($P>0.05$). The production of protein was found to be higher in nutrient broth culture at both extraction temperatures. This possibly could be due to

more capsular materials leached into the liquid environment.

Table 6.2 Effect of temperature on the yield of protein and sialic acid in the capsular material of *P. haemolytica* A2.

Culture media[a]	Treatments					
	Pre-extraction		Post extraction			
	37°C	41°C	37°C		41°C	
	V	V	P	S	P	S
Nutrient Broth	6.4×10^8	6.4×10^8	1.7	0.06	1.73	0.06
Blood Agar	2.1×10^{11}	1.3×10^{11}	1.55	0.06	1.51	0.06

V - viable counts (cfu/ml)
P - Protein concentration (mg/ml)
S - Sialic acid concentration (mg/ml)
a - average of 3 trials

6.2.4 Discussion

Capsular material has been shown to be produced optimally from 6 h cultures of *P. haemolytica* A2 in nutrient broth and the yield of protein and sialic acid composition is not affected by either extraction temperature, 37° C or 41° C. Gentry *et al.* (1981) also demonstrated that capsular material may be removed from young

cultures of *P. haemolytica* A1 by 1 h incubation of PBSS suspended cells at 41 °C. They suggested with this treatment there was a little loss of cell viability until the extraction temperature reached 45℃. At higher temperatures of 45℃ or 56℃ Gentry *et al.* (1981) demonstrated that capsular extracts prepared in water contained less protein than did extracts prepared from the same suspension at 37°C or 41°C, possibly due to the breakdown at the higher temperatures of protein into polypeptides too small to be recognized by the protein assay. They also showed that there was increase in cell lysis and leakage of PBSS suspensions incubated at 45°C or 56°C than those incubated at 41°C or less.

This study has indicated that optimal production of capsular material could be obtained from cells grown in nutrient broth in the early logarithmic phase cultures (6 hours) incubated at 37℃. The capsular material was optimally extracted with minimal cell destruction in PBS suspension incubating at 37°C for 1 hour.

CHAPTER 7.0 THE IMMUNOCHEMICAL ANALYSIS OF OMP-PS COMPLEX OF *P. HAEMOLYTICA* A2 AND ITS IMMUNOGENICITY IN MICE

Capsular polysaccharides (PS) are recognised as important attributes of pneumonia inducing bacteria. Capsular polysaccharides of *N. meningitidis* group B and *E. coli* K1 have been purified and characterised physico-chemically, biologically and immunologically (Kasper *et al.*, 1973; Robbins *et al.*, 1974; Bhattacharjee *et al.*, 1975) and their role in the mediation of pneumonia has been described (Lifely *et al.*, 1984)). *P. haemolytica* A2 strains have been shown to produce PS (Gilmour *et al.*, 1985; Adlam *et al.*, 1987), but there is a descrepancy with respect to their physico-chemical, biological and immunological characteristics as reported by different workers (Gilmour *et al.*, 1979; Donachie *et al.*, 1989).

P. haemolytica A2, the most commonly isolated serotype from sheep pasteurellosis has a capsular structure identical to the capsular polysaccharides of the human meningitis organisms *N. meningitidis* group B and *E. coli* K1. The polymer is a colominic acid of an α-(2$\rightarrow$8)-linked heteropolymer of N-acetyl neuraminic acid (NANA). This capsular structure is known to be an extremely poor immunogen and it

has been suggested that immune tolerance, loose configuration and host neuraminidases allow organisms with such PS to evade detection by host defences (Lifely *et al.*, 1987).

Attempts to induce immunity to group B *meningococci* and *E. coli* K1 have limited success because polymers of an α-(2→8)-linked NANA, alone or complexed to outer membrane proteins, induced low transient levels of IgM antibodies (Wyle *et al.*, 1972; Lifely *et al.*, 1991). However, purified B polysaccharides noncovalently complexed to outer membrane proteins of *N. meningitidis* have shown promising results when tested for their immunogenicity in mice (Peppler *et al.*, 1982; Zollinger *et al.*, 1978). The availability of monoclonal antibodies with specificity for the capsule antigen of *N. meningitidis* group B and a polysaccharide complexed to outer membrane meningococcal vaccine prompted research to evaluate its efficacy in mice against *P. haemolytica* A2 challenge.

This study reports purification and characterisation of purified OMP-PS complex material from culture supernatant of *P. haemolytica* serotype A2 and its contribution as vaccine component against pasteurellosis in mice and sheep.

7.1 The chemical anaysis of outer-membrane protein-polysaccharides (OMP-PS) of *P. haemolytica* A2

7.1.1 Introduction

Capsule preparations of many Gram-negative bacteria can be hydrolysed and the components can be isolated by centrifugation and chromatography (Adlam *et al.*, 1985). The capsule isolated can then be fractionated and analysed. This section describes the isolation, fractionation and analyses of the OMP- polysaccharide complex of *P. haemolytica* A2.

7.1.2 Materials and Methods

The crude complexes were chromatographed on a Sepharose CL-2B column as described in the general Materials and Methods. The ELISA, PAGE and dot blotting procedures are as described in Chapter 3. Sheep antiserum raised against A2 SSE was used at dilution 1 in 500 as the detecting antiserum. Convalescent sheep serum from sheep recovered from experimental pasteurellosis was used at dilution 1 in 50 as the positive control serum.

The complex was analysed for LPS, nucleic acid, protein and sialic acid by the method given in the general Materials and Methods.

7.1.3 Results

Yield of OMP-PS complex: Using the purification method as described in the Figure 3.1, the yield of crude OMP-PS complex of *P. haemolytica* A2 (strain E0-200) per litre of culture supernate was 0.12g.

When the complex was further purified (Figure 3.2), the yield of purified OMP-PS complex was 11.4 mg (9.5% by weight) of the crude complex. The protein and sialic acid (carbohydrate) composition of OMP-PS complex are shown in Table 7.1.

The protein to carbohydrate ratio was 4:1. No attempt was made in these experiments to optimise yields of this complex by for example, alterations of bacterial growth conditions or cetavlon concentration.

Table 7.1 Protein and sialic acid determined in a preparation of OMP-PS complex of *P. haemolytica* A2.

Preparation	Protein (mg/ml)	Sialic acid (mg/ml)	Ratio of protein/ sialic acid
Crude complex	1.1	0.72	1.5
Purified complex (V_O from Sepharose CL-2B)	0.08	0.02	4.0

Physical and chemical analysis: The purified OMP-PS polymer was a white powder and soluble in water. The molecular weight was in excess of 10^6. Gel filtration of the crude complex in Sepharose CL-2B removes low-molecular weight nucleic acids and salts which appear in the total volume (V_T) of the column as well as polysaccharides not associated with the complex at the void volume (V_O). The purified complex (void volume peak) was easily sterile filtered through 0.45 μm and 0.2 μm membranes (Millipore, UK).

Chemical analysis of the crude and purified polymer revealed very little LPS present (0.06% of total amount of complex) when analysed with kinetic turbidimetric assay method using Limulus Amoebocyte Lysate (LAL).

Column chromatography: The elution patterns obtained when crude complex samples were chromatographed on Sepharose CL-2B are shown in Figure 7.1. The fractions were assayed for sialic acid and protein composition.

When assayed for sialic acid and noncovalently bound protein, the crude complex was shown to elute from the column fraction numbers 18-24. Only a small fraction of the sialic acid content is found in the earlier fraction. The greater proportion is found in the later fraction, which does not show any antigenic activity (results not shown).

Dot blotting results using monoclonal antibodies with specificities for *N. meningitidis* group B capsule and polyclonal antibodies from purified OMP-PS of *P. haemolytica* A2 vaccine showed that there is strong reaction with the main fraction of the purified material (Figure 7.2).

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of both crude and purified OMP-PS complex is presented in Figure 7.3 (Lane 2 and 3, respectively). For comparison, the SDS-PAGE pattern of the envelope of the *P. haemolytica* A2 grown in nutrient broth is also shown (Figure 7.3, Lane 4). Fewer protein bands were present in the crude OMP-PS complex, with 2 major bands at 30 kDa and 42 kDa were present. One major band at 42 kDa is present in the purified fraction.

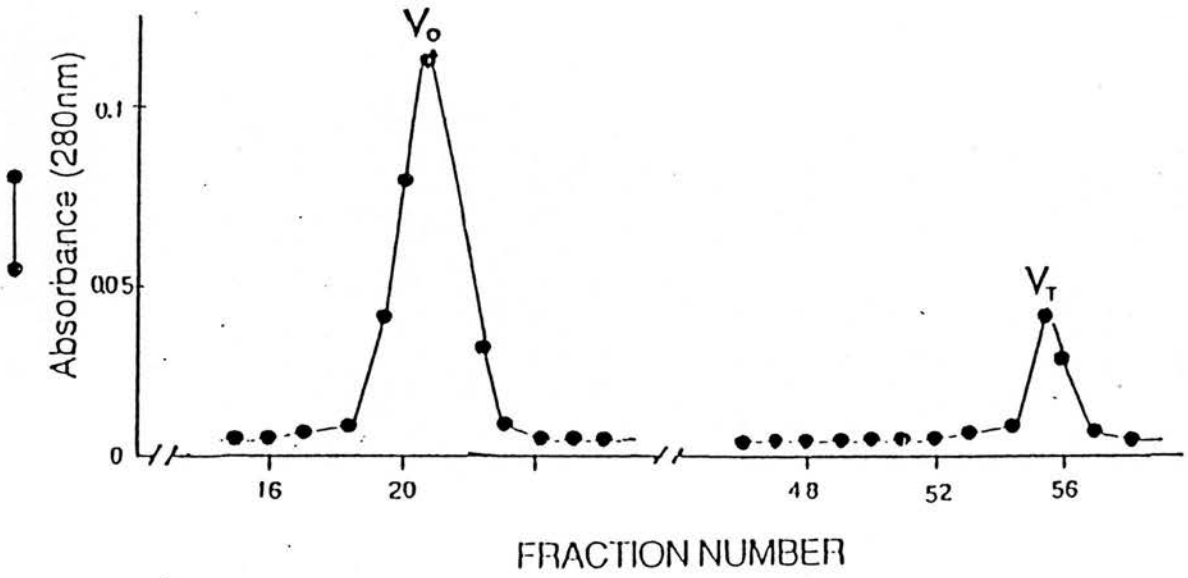

Figure 7.1 **Elution profile of the cetavlon precipitated capsular materials of *P. haemolytica* A2 on Sepharose CL-2B . Fractions (60 x 9 ml) were eluted with ammonium sulphate buffer and assayed for sialic acid and protein.**

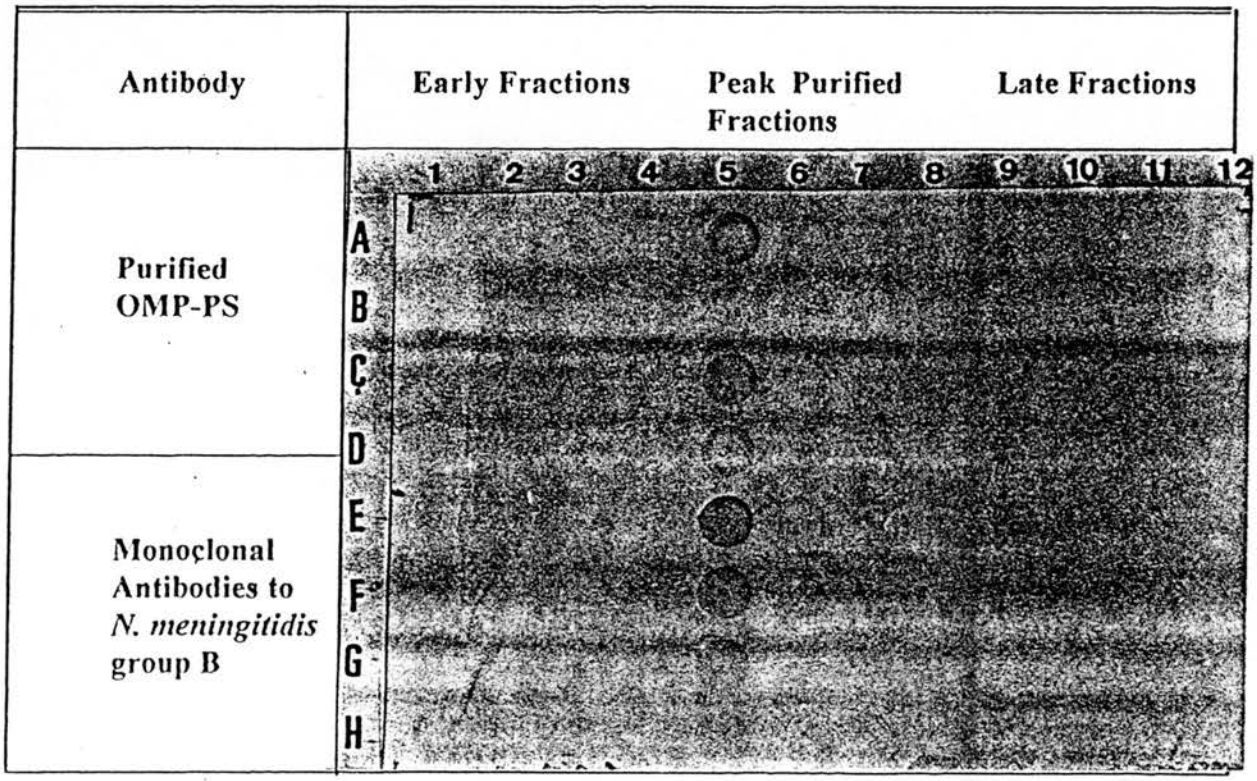

Figure 7.2. Dotblot of different fractions of purified OMP-PS complex material of *P. haemolytica* A2 from Sepharose CL-2B column reacted with antisera raised against a) Purified OMP-PS vaccine, Grid A5-D5; b) Monoclonal antibodies to *N. meningitidis* group B, Grid E5-H5.

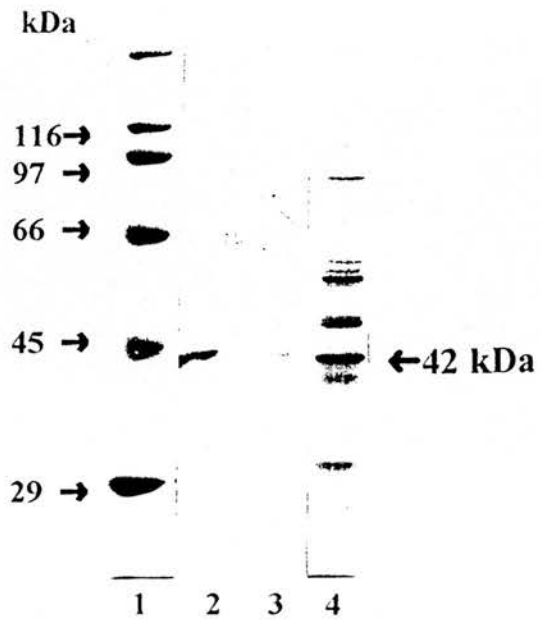

Figure 7.3 SDS- PAGE of *P. haemolytica* A2 outer membrane protein complexed to polysaccharide obtained in the purification desribed in Fig. 4.1. Lane 2 contained a crude OMP-PS and lane 3 contained a purified OMP-PS complex material. Lane 4 contained A2 envelope grown in nutrient broth. Molecular mass markers are shown in lane 1. Each lane contained approx. 20 μg of protein. The 42 kDa major OMP is arrowed.

7.1.4 Discussion

This compares with the outer membrane protein profile obtained from the envelope of *P. haemolytica* A2 whereby the major bands at 30 kDa and 42 kDa were present (Donachie & Gilmour, 1988). Squire *et al.* (1984) also demonstrated that *P. haemolytica* A1 contains major outer membrane proteins of similar molecular mass.

From the results, it can be proposed that the purified complex is composed of mainly carbohydrate, a proportion of outer membrane protein and minimal amounts of LPS and nucleic acid.

7.2 The immunogenicity of OMP-PS of *N. meningitidis* group B against *P. haemolytica* A2 challenge in mice

7.2.1 Introduction

Vaccines containing antigens of *P. haemolytica* serotype A2 have been comparatively poor immunogens in mice, rabbits and sheep (Gilmour *et al.*, 1983; Evans *et al.*, 1979; Donachie *et al.*, 1988). A high degree of immunity against A2 serotype has been reported only for lambs recovered from experimentally induced pasteurellosis (Donachie *et al.*, 1986). Suboptimal efficacy of some *Pasteurella* vaccines against experimental A2 challenge may be explained by the limited range of

antigens on the surface of bacterial cells or the antigens may not be presented in a right conformation to the host immune system.

In *N. meningitidis* group B and *E. coli* K1 the capsule has been identified as a virulence factor and a protective antigen (Schiffer *et al.*, 1976). The murine monoclonal antibodies with *N. meningitidis* group B specificity has been shown to protect mice against challenge (Moreno *et al.*, 1983). *P. haemolytica* serotype A2, the most commonly isolated serotype from pasteurellosis cases in sheep has a capsular structure identical to *N. meningitidis* group B (Adlam *et al.*, 1987). The availability of monoclonal antibodies with specificity for the capsule antigen of *N. meningitidis* group B and a polysaccharide-outer membrane protein complex meningococcal vaccine prompted an examination of the contribution to immunity made by antibodies to *P. haemolytica* A2 capsule.

Although not a natural host, the septicaemic mouse model for pasteurellosis (Evans & Wells, 1979) offers a mean of assessing the efficacy of vaccines and the potential of antibody for passive protection.

7.2.2 Materials and Methods

P. haemolytica A2 (strain A713) was grown in nutrient broth (Gibco No. 2) for 18 hours at 37°C. Cells for mouse challenge were pelleted by centrifugation at

3,000 *g* for 20 minutes before resuspension in phosphate buffered solution (PBS), pH 7.4. Cells for immunoblotting were centrifuged as above but resuspended in distilled water. Whole cell preparations of *N. meningitidis* and *P. haemolytica* were separated by SDS-PAGE and transferred to Immobilon P (Millipore). The blots were probed with mouse antisera and sheep anti mouse IgG-HRP conjugate (SAPU) was used to detect bound mouse antibodies. For bactericidal and opsonophagocytosis assays, the methods described by Sutherland, (1988) and Donachie *et. al.* (1986) were used.

N. meningitidis group B type 6 (srain CN7622) was grown in 5% sheep blood agar for 18 hours at 37°C, harvested in PBS, pelleted by centrifugation at 3,000 *g* for 20 minutes before resuspension in distilled water.

Mice immunisation and challenge: C57 black mice (6 weeks old) were passively immunised with monoclonal antibodies or control positive or negative sera one hour before challenge. For vaccination, meningococcal vaccine (Wellcome Biotech) at 1μg/dose was given intraperitoneally twenty and ten days before challenge. For challenge the mice were injected intraperitoneally with $10LD_{50}$ of *P. haemolytica* A2 in 80% hog gastric mucin. Five hours after challenge, the mice were humanely killed, the livers removed, macerated and the number of viable organisms estimated by titration.

7.2.3 Results

The activity of the eight monoclonal antibodies in the IHA test, bactericidal and opsonophagocytic assays are shown in Table 7.2.

Seven antibodies demonstrated an IHA titre with serotype A2 antigen and the titres ranged from 1/80 to 1/320. A low titre cross reaction was observed with serotype T10. Little or no activity was observed with any of the antibodies in the bactericidal or opsonophagocytic assays.

Two antibodies with IHA activity (34A10 and 62A13) were assessed in a passive mouse protection test where the mice were challenged with serotype A2 (Figure 7.3). The results show that significant protection was observed on two occasions for antibody 62A13 ($P<0.05$) and on one occasion for antibody 34A10.

The contribution of capsular antigen to immunity and the efficacy of direct immunisation was assessed by immunising mice with a meningococcal polysaccharide-OMP vaccine and challenging with serotype A2. The results of this immunisation are shown in Figure 7.4. Significant protection was conferred on mice by the vaccine ($P<0.05$). Assay of the serum antibodies of immunised mice by the IHA test indicated high titres in a number of animals to serotype A2 capsule.

Table 7.2. **The IHA titre, bactericidal activity (%K) and opsonophagocytic (%P) of monoclonal antibodies specific for *N. meningitidis* group B capsule polysaccharide.**

mAb (1mg/ml)	IHA (recip. titre)	%K	%P
14A2	20	<5	ND
22A2	320	<5	ND
32A1	0	<5	7
34A10	80	<5	ND
35A1	320	<5	ND
36A10	320	<5	ND
62A13	160	<5	ND
85A5	80	<5	ND

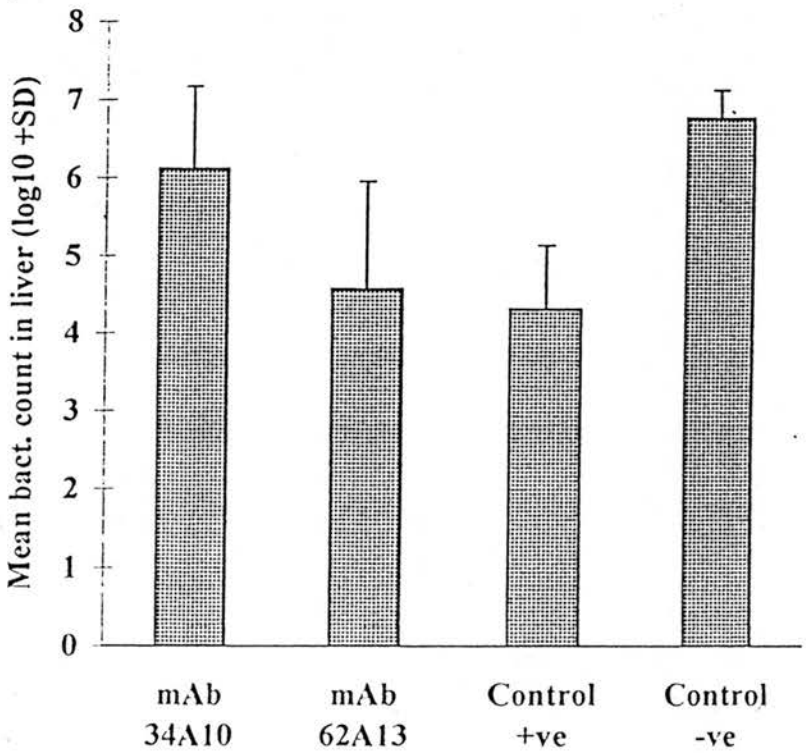

Fig. 7.4. Passive protection of mice to *P. haemolytica* A2 challenge by monoclonal antibodies specific for *N. meningitidis* group B capsule polysaccharide

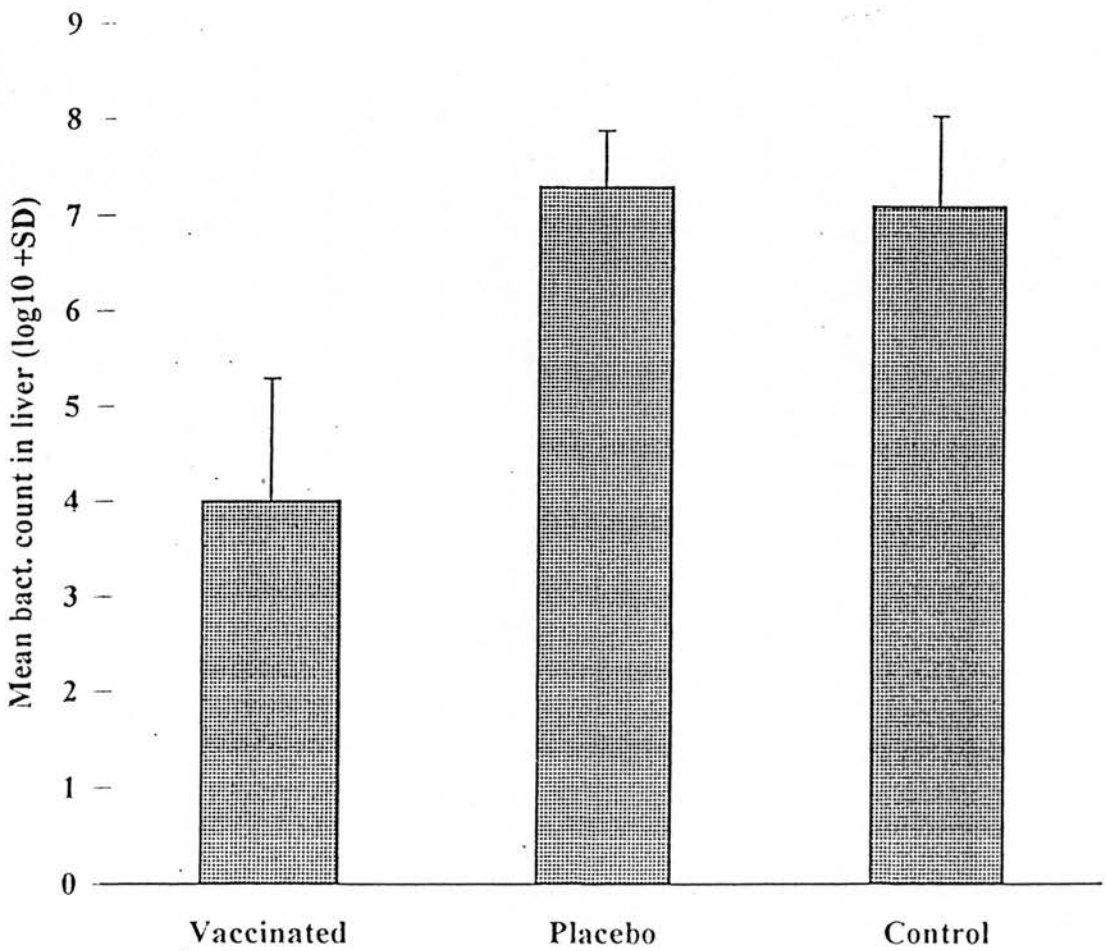

Fig. 7.5. Protection of mice to *P. haemolytica* A2 challenge by immunisation with Meningococcal group B vaccine

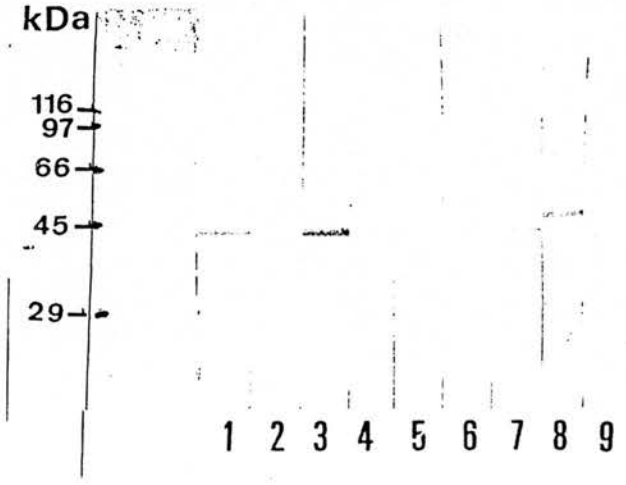

Figure 7.6. Immunoblot of *N. meningitidis* group B whole cells probed as follows: Lane 1-7, mouse antisera raised against Meningococcal group B vaccine; Lane 8, mouse antisera raised against *P. haemolytica* A2; Lane 9, serum from nonimmunised mice

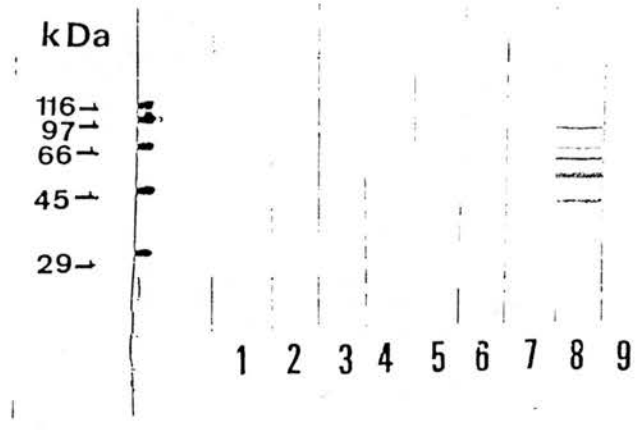

Figure 7.7. Immunoblot of *P. haemolytica* A2 whole cells probed as follows: Lane 1-7, mouse antisera raised against Meningococcal group B vaccine; Lane 8, mouse antisera raised against *P. haemolytica* A2; Lane 9, serum from nonimmunised mice

Sera from vaccinated mice were examined in immunoblots against *N. meningitidis* and *P. haemolytica* whole cells to determine the antibody response to cellular proteins (Figure 7.6 and 7.7). Very little response was observed to *P. haemolytica* antigens but a strong response was observed to *N. meningitidis* antigen of around 43 kDa.

7.2.4 Discussion

Monoclonal antibodies with specificities for *N. meningitidis* group B capsule protected mice against *P. haemolytica* A2 challenge. Similar protection has previously been shown against *E.coli* K1 challenge (Moreno *et al.*, 1983). The results reported are in agreement with that of Moreno *et al.* (1983) who succesfully produced murine monoclonal antibodies with group B specifities and showed their protective value in mice against *N. meningitidis* challenge. It also indicated the shared protective antigens in the capsular polysaccharide structure of *N. meningitidis* group B and *E. coli* K1. The poor immunogenicity of purified group B polysaccharide is well documented (Frasch *et al.*, 1978; Gotschlich *et al.*, 1978). The polysaccharide does not induce specific antibodies, and the response against whole bacteria, or the polysaccharide in a complexed form, is relatively short lived and consists almost exclusively of IgM antibodies (Zollinger *et al.*, 1978, 1979). It has been suggested that immune tolerance, loose configuration and host neuraminidases may all contribute to this (Lifely *et al.*, 1987). These factors may also explain the comparatively poor immunogens of serotype

A2 capsular antigen in mice (Gilmour *et al.*, 1983).

In this study the protective value of monoclonal antibody to group B polysaccharide was demonstrated. The results confirm the importance of antibodies to colominic acid capsules in protection.

Previous attempts to immunise mice with B polysaccharides covalently complexed to outer membrane proteins were not succesful (Jennings *et al.*, 1981). In lambs, the A2 capsular antigen did not induce an antibody response (Donachie *et al.*, 1986). In this study the results has shown that meningococcal vaccine protected mice against A2 infection. The findings suggest that the antigens are presented in the right way thus increasing its immunogenicity in mice. The results in this study are the first demonstration of direct immunisation against A2 infection in the mouse model, however, the mechanism of protection is unclear as both bactericidal and opsonophagocytic activity could not be demonstrated.

P. haemolytica vaccines with a similar composition to the meningococcal vaccine should be evaluated in ruminants for efficacy against experimental pasteurellosis.

7.3 The immunogenicity of OMP-PS of *P. haemolytica* A2 in mice

7.3.1 Introduction

Previous experiments have shown that mice were protected when passively immunised with monoclonal antibodies with specificity for capsule antigen of *N. meningitidis* group B and a polysaccharide-outer membrane protein complex meningococcal vaccine. Further immunisation of the mice with *N. meningitidis* capsule-OMP complex also confers protection in the mice. Since the A2 capsule antigen is identical to *N. meningitidis* it follows that the A2 capsule is potentially an important protective antigen.

In the experiments in Chapter 6, the purified capsular complex material was extracted and chemically characterised. This experiment was conducted to demonstrate the protection afforded by vaccination with this purified A2 capsular polysaccharide-OMP A2 vaccine in mice.

7.3.2 Materials and Methods

Mice immunisation and challenge

Experiment 1: Fifty mice were randomly allocated to five groups of 10 mice each. The layout of the experiment is shown in Table 7.3. Details of the vaccine

preparations are given in the general Materials and Methods.

Experiment 2: In this experiment, groups of mice immunised with Ovipast^{R} vaccine and leukotoxin materials were included in the experimental design to determine the protective value of IRP and leukotoxin antigen in mice. The layout of the experiment is shown in Table 7.1. Details of the vaccine preparations are given in the general Materials and Methods.

Three weeks after the second vaccination, inocula of *P. haemolytica* A2 in gastric mucin were prepared and used to challenge the mice. Viable counts were performed on the livers of invidual mouse six hours after challenge. Viable counts are expressed as $\log_{10}$ cfu.

Serology: Immunoblotting and IHA tests were performed as described in the general Materials and Methods.

7.3.3 Results

The results of experiment 1 are summarised in Table 7.3. All the vaccinated mice had lower liver counts than did the unvaccinated controls but only the mice vaccinated with purified A2 OMP-PS complex and meningococcal group B vaccines were significantly different ($P<0.005$) from the controls counts. There is some

indication that the degree of protection conferred by the purified A2 OMP-PS complex vaccine is better (1.3 cfu), although not significantly different from the meningococcal B vaccine (1.8 cfu).

The results of experiment 2 are summarised in Table 7.4. The experiment again demonstrated that all vaccinated mice had lower liver counts than did the unvaccinated controls but only the mice vaccinated with purified OMP-PS complex, meningococcal group B vaccine, Ovipast and a mixed purified OMP-PS vaccines were significantly different from the control counts ($p<0.004$). The protective value of the Ovipast vaccine was similar when the vaccine was enriched with the purified OMP-PS A2 complex material. In contrast, the leukotoxin vaccine did not protect the mice. However, when the purified OMP-PS A2 complex material was added to it, significant protection of mice was demonstrated ($p<0.004$).

Serology: The results of immunoblotting of sera from all the groups of vaccinated and control mice against *P. haemolytica* A2 antigens are shown in Figure 7.8 and Figure 7.9. The IHA test gave inconclusive results in that there was no measureable IHA titre in all groups but some individual mice immunised with purified OMP-PS A2 vaccine showed IHA titres ranged from 1/4 to 1/16.

Immunization with the whole cell vaccines or the heated culture supernatant elicited responses to numerous antigens. Strongly reacting bands were seen

at major OMP bands of molecular mass 30 kDa and 42 kDa. Crude OMP-PS and purified OMP-PS vaccines elicited responses to the major OMPs but not to the minor bands seen with whole cell vaccinations. Antisera raised against leukotoxin did not contain specificities against the OMPs and even when OMP-PS were added to it.

Table 7.3 Counts[a] of *P. haemolytica* A2 in the livers of unvaccinated control mice and mice vaccinated with *P. haemolytica* A2 antigens after challenge[b].

Group	Vaccine	Vaccine Dose	Mean count in liver after 6 h (cfu)[a]	p*
1	Heat killed organisms	2.9×10^8 cfu/ml	4.499	NS
2	Heated culture extract	2.9×10^8 cfu/ml	4.298	NS
3	Crude OMP-PS extract	10 μg sialic	4.147	NS
4	Purified OMP-PS extract	10 μg sialic	1.253	<0.002
5	Meningococcal Gp.B vaccine	1 μg	1.803	<0.005
6	Unvaccinated controls	-	5.494	-

a - Counts = cfu expressed as $\log_{10}$ values
b - Challenge dose (1.02×10^7 cfu)
* - When compared with the control group in the Mann-Whitney Ranking Test

Table 7.4. Counts[a] of *P. haemolytica* A2 in the livers of unvaccinated control mice and mice vaccinated with *P. haemolytica* A2 antigens after challenge[b].

Group	Vaccine	Vaccine Dose	Mean count in liver after 6 h (cfu)[a]	p*
1	Heat killed organisms	2.9 x 10^8 cfu/ml	5.901	NS
2	Heated culture extract	2.9 x 10^8 cfu/ml	5.595	NS
3	Crude OMP-PS extract	10 μg sialic	5.400	NS
4	Purified OMP-PS extract	10 μg sialic	1.857	<0.002
5	Meningococcal Gr.B vaccine	1 μg	1.063	<0.001
6	Ovipast	2 ml	1.570	<0.002
7	Ovipast and OMP-PS extract	2 ml + 10 μg sialic	2.860	<0.002
8	Leukotoxin	2 ml	5.847	NS
9	Leukotoxin and Purified OMP-PS extract	2 ml + 10 μg sialic	2.904	<0.004
10	Unvaccinated control	-	7.219	-

a - Counts = cfu expressed as $\log_{10}$ values
b - Challenge dose (1.02 x 10^7 cfu)
* - When compared with the control group in the Mann-Whitney Ranking Test

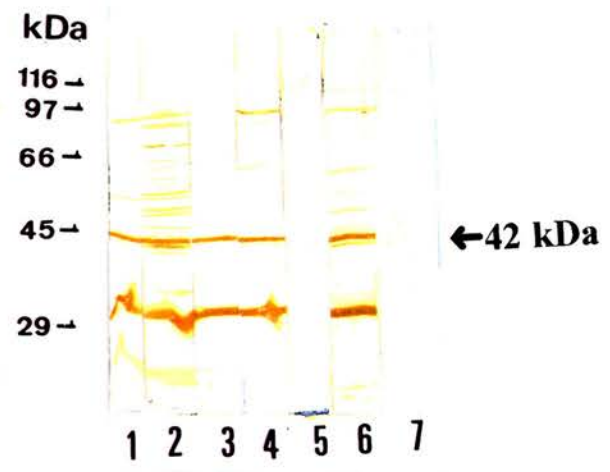

Figure 7.8. **Immunoblot of *P. haemolytica* A2 whole cells reacted with the following serum pools;**

Lane numbers:

1. **Heat killed organisms**
2. **Heated culture extract**
3. **Crude OMP-PS**
4. **Purified OMP-PS**
5. **Meningococcal vaccine**
6. **Sera from sheep recovered from A2 infection (+ve control)**
7. **Non-immunised mouse**

Figure 7.9. Immunoblot of *P. haemolytica* A2 whole cells reacted with the following serum pools;

Lane numbers:

1. Heat killed organisms
2. Heated culture extract
3. Crude OMP-PS
4. Purified OMP-PS
5. Meningococcal vaccine
6. Ovipast
7. Ovipast + Purified OMP-PS
8. Leukotoxin
9. Leukotoxin + Purified OMP-PS
10. Sera from sheep recovered from A2 infection (+ve control)
11. Non-immunised mouse

7.3.4 Discussion

The outer membrane protein-polysaccharide complex of *N. meningitidis* group B protected mice against challenge with *P. haemolyica* A2 (Chapter 7.2). Further work was carried out to obtain similar preparations of OMP-PS from *P. haemolytica* A2. In this study it was shown that that the antigen (or antigens) responsible for mouse protection are present in the purified OMP-PS complex of *P. haemolytica* A2 and *N. meningitidis* group B and Ovipast^{R} vaccine. The purified complex was shown to be predominantly polysaccharide in nature compared to the protein rich composition of crude complex. This was shown in the immunoblotting results where the crude OMP-PS material demonstrated the presence of the same protein components and responses to these are detected with the antisera raised against the crude preparation vaccinates but the mice were not protected. Therefore the difference must be in the response to the polysaccharide antigen.

Immunoblotting and PAGE examination of the protein profile has indicated that the protein of molecular weight 42 kDa is an important antigen. This is the major outer membrane protein band as been reported by other investigators (Squire *et al.*, 1984; Donachie *et al.*, 1988).

The results also demonstrated that Ovipast^{R} vaccine alone or in combination with purified OMP-PS complex has been shown to confer immunity to mice

against A2 challenge as indicated by low total viable counts. In contrast, no protection was observed in mice vaccinated with leukotoxin. The protection evident in the Ovipast vaccinated mice may be explained by the presence of antigens in the vaccine which are important in the protection while leukotoxin lacks some of these antigens. Immunoblotting results showed that antisera raised against leukotoxin did not contain specificities to OMP. Donachie (1993, unpublished results) also showed that calves responded serologically to vaccination with IRPs but not to leukotoxin. The immunogenicity of A2 IRP antigens has been demonstrated by Gilmour *et al.* (1991) using cell surface extract of cells grown under iron restriction and expressing IRPs. In a series of experiments to test the efficacy of the Ovipast IRP, it has been demonstrated that the vaccine conferred protection on SPF lambs against A2 disease and significantly reduced the incidence of disease, numbers of bacteria recovered and severity of lung lesions (Donachie, personal communication).

This experiment showed that OMP-PS can confer protection in mice and therefore that the protective antigen is present in this extract. Mice immunised with purified OMP-PS complex vaccine of *P. haemolytica* A2 developed humoral antibodies detectable by IHA test and immunoblotting results showed that 42 kDa OMP of *P. haemolytica* A2 seemed to be the immunogenic protein component and therefore most essential for noncovalent binding to the capsular polysaccharide. The IHA antigen in the purified OMP-PS complex material is carbohydrate in nature and is the important antigen in the protection of mice against challenge with *P. haemolytica* A2.

CHAPTER 8.0 THE EFFICACY OF OMP-PS COMPLEX *P. HAEMOLYTICA* A2 VACCINE IN SHEEP

Developments in *Pasteurella* vaccines are moving away from the use of crude whole cell bacterins towards identification and incorporation of specific protective antigens. These latter antigens have been used to enrich existing vaccines by culture modification or by addition of purified components.The vaccines which protected mice against *P. haemolytica* infection also protected SPF lambs, but not against serotype A2 infection (Gilmour *et al.*, 1979). The antigens from serotype A2 tend to be weak immunogens. Sialic acid is a major component of A2 capsule and the failure of previous attempts to immunise mice and sheep with capsular vaccines against A2 challenge could be due to poor anti-capsular response elicited by the vaccines. These sialic acid polysaccharides in complex with outer membrane protein have shown promising results when tested for their immunogenicity in mice (Chapter 7).

The following experiments were conducted to study the immunogenicity of crude and purified OMP-PS complex in conventional sheep and to determine the possible contribution of this candidate antigen to *Pasteurella* vaccines.

8.2 Materials and methods

Vaccine preparations and formulation: The vaccines were prepared as described in the general Materials and Methods. The vaccines contained lyophilised crude or purified OMP-PS complex of *P. haemolytica* A2; resuspended in distilled water to contain a standard concentration of 10 μg/ml sialic acid. Alumunium hydroxide (Alhydrogel, Superfos Denmark) was added to all vaccines to a 0.27% final concentration.

Experimental design: Three groups of 4 lambs aged 3 months and 3 groups of ewes aged 2 years were used in this experiment. The sheep had been clinically healthy and tested negative for *Pasteurella* spp. The ewes and the lambs were randomly allocated to vaccine groups. Two vaccine preparations consisting of crude and purified OMP-PS vaccines complex were administered to two groups in each age group. The animals were vaccinated subcutaneously (2ml dose) on the lateral aspect of the middle third of the neck at day 0 and booster given 4 weeks later. The remaining group of lambs and ewes were sham-vaccinated and served as controls. The animals were grazed together at pasture raised at Moredun for the duration of the experiment.

Passive mouse protection test: Thirty mice were used and divided into three groups of ten. The mice in group 1 were inoculated intraperitoneally with 0.5 ml of sheep sera raised against purified OMP-PS complex, the mice in group 2 were

inoculated intraperitoneally with 0.5 ml of sheep sera raised against crude OMP-PS material and the last group were sham-vaccinated controls. One hour after inoculation all the mice were challenged intraperitoneally with $2.1x10^7$ cfu/ml *P. haemolytica* A2 in gastric mucin. Six hours after challenged all the mice were killed and viable counts were performed as described in general Materials and Methods.

Sheep sera: The animals were bled for serology by jugular venepuncture (10 ml vacutainers) on day 0 and at weekly intervals for 6 weeks. A pool of sera from SPF lambs recovered from experimental *P. haemolytica* infection served as a reference positive serum in the immunoblotting experiments (Donachie *et al.*, 1988).

Serological tests: IHA test, SDS-PAGE and immunoblotting were performed according to the method described in the general Materials and Methods.

8.3 Results

The serum antibody titres of the adult sheep and lambs vaccinated with the OMP-PS complex vaccine and the control groups (sham-vaccinated), measured in the IHA test, plotted against time are shown in Fig.8.1-8.2. The purified OMP-PS vaccine stimulated a highly significant ($P<0.05$) IHA antibody response to *P. haemolytica* A2 in the adult sheep when compared to the crude OMP-PS vaccine and sham-vaccinated group. Antibody levels increased quickly to a titre of 1:16 a week after primary vaccination and again increased to a mean titre >1:96 after second vaccination and then

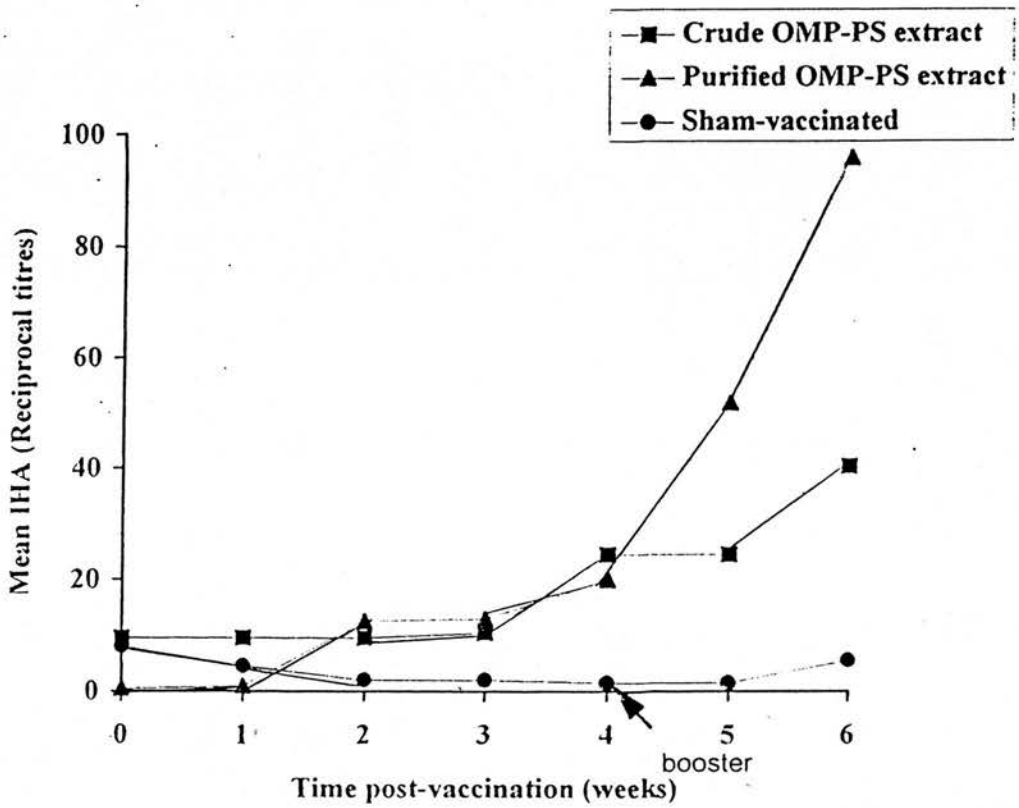

Fig. 8.1. Mean IHA antibody response to *P. haemolytica* A2 capsule antigen for adult sheep vaccinated with crude OMP-PS and purified OMP-PS extract vaccines

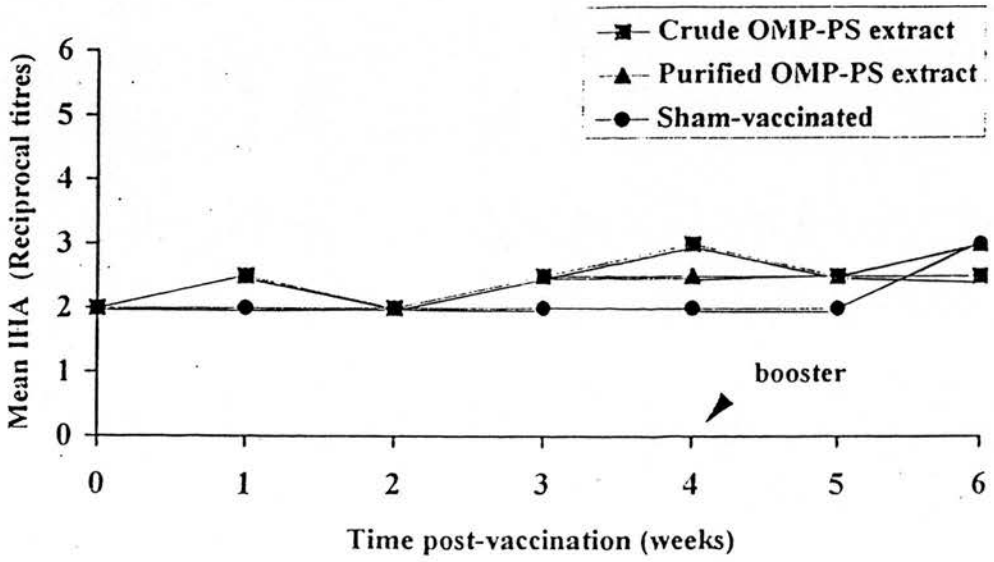

Fig. 8.2. Mean IHA antibody response to *P. haemolytica* A2 capsule antigen for lambs vaccinated with crude OMP-PS and purified OMP-PS extract vaccines

maintained thereafter. The adult sheep that were vaccinated with the crude OMP-PS material had low levels of titres (1:8) until week 3 and showed increased titre only after secondary vaccination. Lambs appeared to have no response to the vaccines. The mean serum antibody titre remained low (<1:4) throughout the study period.

The superior serological response in the group of ewes which received the purified material compared to the crude complex vaccine group was maintained throughout the subsequent six weeks periods. The mean serum antibody titres of the sheep in the sham-vaccinated control groups remained almost constant (<1:8) throughout the experiment.

The results of the mouse passive protection test are shown in Table 8.1. The viable counts in the livers of mice immunised with the purified OMP-PS complex vaccine were significantly lower than the counts in the other 2 groups ($p<0.004$) indicating good protection afforded by the vaccine.

The results of immunoblotting of sera from all the groups of vaccinated and control sheep against *P. haemolytica* A2 whole cells antigens are shown in Figure 8.3. Sera from vaccinated animals recognize numerous antigens in the A2 whole cells indicating successful immunisation. There are strong reactions with all the sera from

Table 8.1 Passive protection of mice against *P. haemolytica* A2 challenge with sheep antisera raised against OMP-PS extracts of *P. haemolytica* A2

Group	Sheep Antisera	Dose/ mouse	Mean count in liver after 6h (cfu)[a]	p*
1	Purified OMP-PS	0.5 ml	1.500±0.006	<0.004
2	Crude OMP-PS	0.5 ml	4.082±0.008	NS
3	Sham-vaccinated	0.5 ml	4.809±0.003	NS

a - Counts = cfu expressed as $\log_{10}$ values
b - Challenge dose (2.1×10^7 cfu)
* - When compared with the control group in the Mann-Whitney Ranking Test

vaccinated group against the protein in the 42 kDa region. Although a strong reaction in the sera from the vaccinated sheep in the 42 kDa was also demonstrated by the sera from crude OMP-PS group but the sera did not protect the mice in passive protection test. Instead the mice immunised with the sera from sheep vaccinated with the purified OMP-PS were highly protected. Therefore the polysaccharide material which was present in the purified complex was important in this protection.

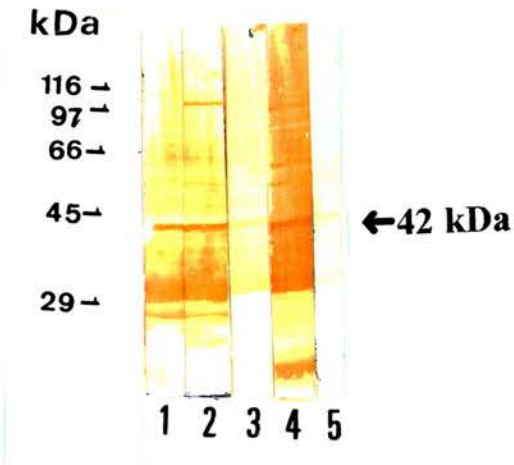

Figure 8.1. **Immunoblot of *P. haemolytica* A2 whole cells reacted with the following sheep sera;**

Lane numbers:

1. **Crude OMP-PS**
2. **Purified OMP-PS**
3. **Sham-vaccinated**
4. **Sera from sheep recovered from A2 infection (+ve control)**
5. **Unvaccinated sheep**

8.4 Discussion

The results of this trial indicate that the purified preparation of OMP-PS complex vaccine was most successful in enhancing the serological response. The complex was immunogenic in adult sheep with induction of humoral anti-capsular antibody in IHA test and with OMP in immunoblotting.

Previous results indicated that direct immunisation of mice with the complex vaccine demonstrated significant protection against A2 infection. The sera from two-year old sheep immunised with OMP-PS passively protected mice against A2 challenge. Three-month old lambs immunised with the same vaccine did not respond serologically and their sera did not passively protect mice.

The immunogenicity of bacterial cell surface antigens has been studied carefully as they are likely to be those which are important in conferring protection against infection. Although bacterial polysaccharides are often very antigenic, their immunogenicity is dependent on such things as molecular weight, the dose of polysaccharide given and their interaction with the imunological mechanisms of the host (Jann and Jann, 1977). Our study had revealed that A2 capsular polysacharide non covalently bound to OMP was immunogenic in older sheep.

Since sialic acid, the only major amino sugar of *P. haemolytica* A2, is also a major constituent of mammalian cell membranes and many animals glycoprotein, it is possible that genetic immunologic tolerence to this polysaccharide is the cause of the lack of antibody response. The poor antibody responses in the young lambs are likely to be due to self immune tolerance. In man, the polysialidated proteins that are found in the embryonal and newborn brain tissue are identical to the repeating units of sialic acid in *N. meningitidis* group B polysaccharide (Finne *et al.*, 1982, 1983). The situation is possibly the same with the young lambs, consequently the animals are tolerant to this self epitope resulting in poor antibody response to purified OMP-PS of *P. haemolyica* A2 complex vaccine.

The study has demonstrated that immunity and protection in sheep are not confined to vaccination with live organisms, and that outer membrane protein-polysaccharide complexes can act as very effective vaccines in mice and sheep. One should be cautious, however in attempting to extrapolate the protective value of this vaccine in a mouse models compared to sheep models.

CHAPTER 9.0 A STUDY OF MACROPHAGE PHAGOCYTOSIS TO DISTINGUISH BETWEEN ADHERENT AND PHAGOCYTOSED *P. HAEMOLYTICA* A2

9.1 Introduction

Capsular polysaccharides have been extracted from cultures of *P. haemolytica* A2 complexed to outer membrane protein in high molecular weight form and shown to be highly immunogenic in mice and adult sheep (earlier experiments). The capsular polysaccharide is recognised as an important virulence attribute in preventing phagocytosis and bactericidal serum activity.

Serum antibody is thought to exert several functionally significant biologic roles such as bacterial opsonisation, agglutination of particulates, complement activation, neutralisation of bacterial toxins and viruses and lysis of gram-negative bacteria in the presence of complement (Hand *et al.*, 1974). During acute respiratory tract infection, there may be rapid influx of serum immunoglobulins into alveolar spaces, thereby providing increased opportunities for interactions between specific and nonspecific pulmonary defense mechanisms (Hunninghake *et al.*, 1979). The authors also showed that resident alveolar macrophages and PMN from

the peripheral blood are the initial line of cellular host defense in the lower part of the respiratory tract against inhaled bacterial agents. The defense against bacterial pneumonia depends upon the numerical adequacy of phagocytes in the lower respiratory tract, opsonisation of the bacteria, and effective intracellular killing of the bacteria by the phagocytes (Horvitz, 1982). The purpose of this study was to determine phagocytic response of ovine alveolar macrophages and murine peritoneal macrophages to both opsonised and nonopsonised preparations of ultra violet-killed *P. haemolyica* A2 in the present of antibody raised against crude and purified OMP-PS complex antigens of *P. haemolyica* A2.

9.2 Materials and Methods

Bacteria: An aliquot (0.5 ml) of *P. haemolytica* A2 was incubated overnight in 10 ml nutrient broth at 37°C. Bacteria were inactivated by exposing to ultra violet (UV) light for 3 h.

Fluorescence labeling of bacteria: Fluorescein thiocyanate (FITC)-labeling of bacteria was conducted according to the method described by Drevets & Campbell, (1991). Briefly UV-killed *P. haemolytica* were washed in PBS, then labeled with FITC by incubation with 0.1 mg/ml FITC isomer 1 (Sigma Chemical Co., UK) in 0.1 M $NaHCO_3$, pH 9.0 at room temperature for 60 min. Bacteria were pelleted at 3500 *g* for 15 min., then washed free of unbound fluorochrome with PBS and stored frozen at -20°C until used.

Sera: Control normal mouse sera and sheep sera containing antibodies to purified and crude OMP-PS of *P. haemolytica* A2 and *N. meningitidis* group B were obtained from the previous experiments.

Cells: Inflammatory peritoneal macrophages were obtained by injecting mice intraperitoneally with 1 ml sterile 10% peptone water. Cells were harvested 48 h later by peritoneal lavage with 10 ml cold, sterile Hank's solution. The cells were resuspended in Hank's and counted in hemacytometer.

Sheep broncoalveolar macrophages (BAM) were recovered from lungs of slaughtered, apparently healthy animals as described by Sutherland *et al.* (1983). Sheep BAM were centrifuged at 500 *g*, washed twice in Hanks and finally resuspended in Hanks medium + 10 % dimethyl sulphoxide (DMSO) at 1 to 2 x 10^7 viable BAM/ml. BAM were stored in 1 ml aliquots at -70°C until required.

Aliquots of stored BAM were thawed, pooled and made up to a 20 ml volume of BAM suspension with Hanks. BAM were centrifuged at 500 *g* for 10 min and the sedimented cells washed twice in Hanks and resuspended in 4 ml of Hanks medium + 10% FBS. A cell count and viability estimate were carried out. The suspension was adjusted to 2 x 10^6 viable BAM/ml in Hanks + 10% FBS.

An aliquot (0.5 ml) of BAM suspension was added to each polypropylene vial containing *P. haemolytica* A2 organisms which were pre-opsonised with test sera.

Two control samples containing bacteria with FBS also received BAM (non-opsonised controls) and a further two control samples received 0.5 ml of Hanks medium + 10% FBS (bacteria controls).

Phagocytosis assay: Phagocytosis assay was measured using a standard assay as described by Czuprynski *et al.* (1984). Briefly, $2.5x10^6$ macrophages and $2.5x10^7$ FITC-labeled *P. haemolytica* A2 were mixed with the control and test serum and diluted to 1 ml final volume in siliconised 12x7.5 mm bottles. The bottles were incubated at 37°C for 30 min on a rolling shaker. Free bacteria were removed by washing the cells three times with 2ml iced Hank's solution. The cells were resuspended in 1 ml PBS with 5% foetal calf serum and 5 mM glucose. Cells were held on ice prior to examination. After phagocytosis 100 µl aliquots of macrophages in suspension were removed and mixed with ethidium bromide (EB). Then a 10 µl drop was immediately placed on a glass slide, and overlaid with a cover slip. Phagocytosis of FITC-labeled bacteria was visualised with a fluoresence microscope (Olympus PM-20, Japan) using a 520 nm FITC filter under oil immersion (1000 X) and quantified by counting 40 consecutive individual macrophages with one or more associated bacteria.

Photographs were taken through the fluorescence microscope with a Olympus BX-20 camera using Kodak Ectachrome 1600 EES 135 film. Original magnification of photographs was 240 X.

9.3 Results

The group mean of macrophages with internalised and externalised FITC-labeled *P. haemolytica* A2 coated with anticapsular antibodies for each test group are presented in Table 9.1-9.2. The number of *P. haemolytica* A2 organisms inoculated into suspension was 2.5×10^7 cfu/ml. The bacteria to macrophage ratio was approximately 10:1. The mean number of mouse peritoneal macrophages that showed phagocytosis of opsonised bacteria was significantly higher ($p<0.05$) when the antisera used for opsonisation was taken from purified OMP-PS immunised animals ($p<0.01$). The macrophages which were incubated with bacteria and serum raised against crude OMP-PS complex antigens and serum from sham-vaccinated mice were unable to phagocytose bacteria. The bacteria were not opsonised thus were not phagocytosed and a high proportion (72.5%) of the bacteria remained extracellular. In contrast the mouse peritoneal macrophages internalised 84.3% of the bacteria incubated in the serum raised against the purified preparation of OMP-PS complex. When the antisera used for opsonisation was taken from sham-vaccinated mice the phagocytic activity was poor. Eighty five percent of the bacteria remained extracellular.

Plate 9.1 and Plate 9.2 showed FITC-labeled *P. haemolytica* A2, which by themselves fluoresce bright green and then fluoresced a bright orange after the addition of ethidium bromide. Plate 9.3 showed mouse peritoneal macrophages with

Table 9.1. Adhesion versus phagocytosis of *P. haemolytica* A2 by murine peritoneal macrophages.

Sera	Mean no. macrophage with intracellular bacteria	Mean no. macrophage with extracellular bacteria	% Phagocytosis
Purified OMP-PS	33.33±1.7	6.67±1.7	83.33±4.2
Crude OMP-PS	11±2.1	29±2.1	27.5±5.2
Sham-vaccinated	7.33±0.67	32.67±0.67	18.33±1.7

Table 9.2. Adhesion versus phagocytosis of *P. haemolytica* A2 by sheep alveolar macrophages.

Sera	Mean no. macrophage with intracellular bacteria	Mean no macrophage with extracellular bacteria	% Phagocytosis
Purified OMP-PS	27.67±1.5	12.33±1.5	69.19±3.6
Crude OMP-PS	7.33±0.67	32±1.2	20±2.9
Sham-vaccinated	6.33±0.88	33.67±0.88	15.83±2.2

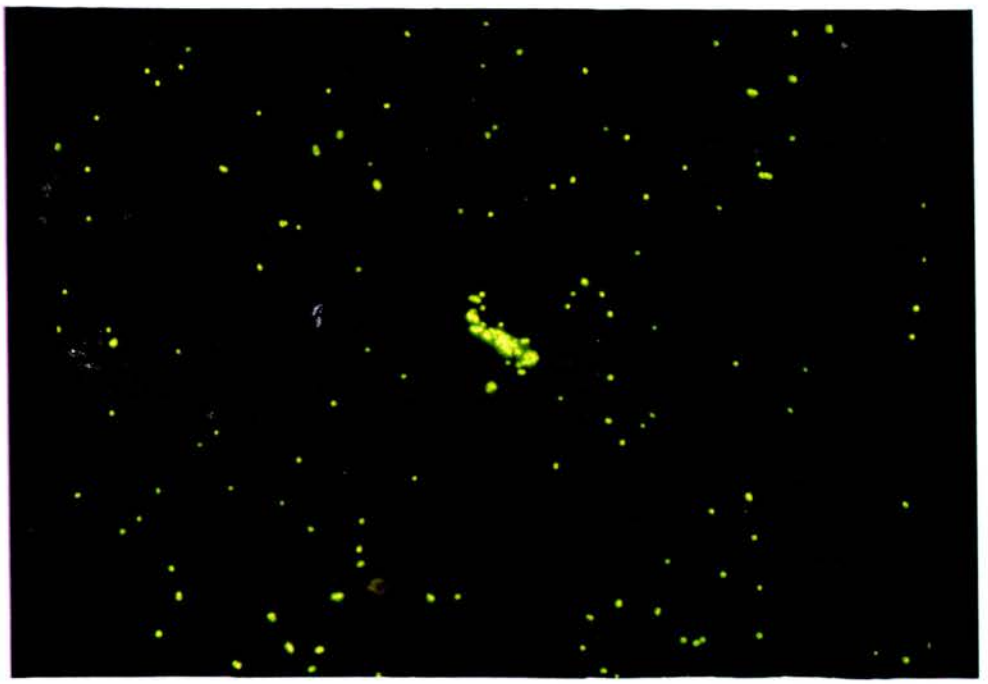

Plate 9.1. **Flourescent antibody reactions. Cells of *P. haemolytica* A2 were treated with antibody conjugated with fluorescein isothiocynate (FITC), which fluoresces green (magnification x 240)**

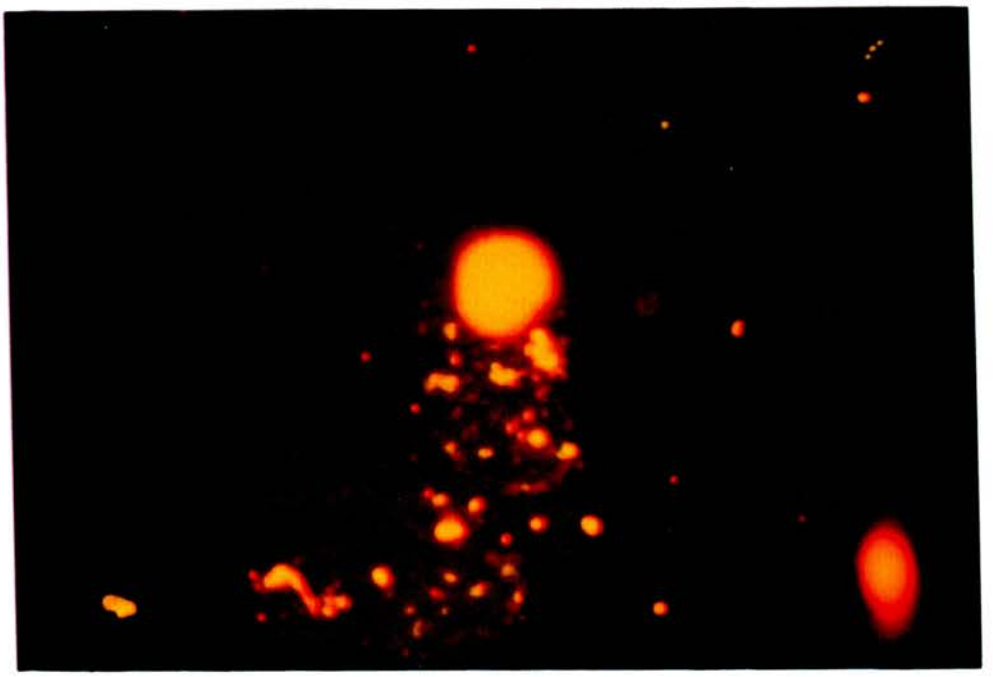

Plate 9.2. **Fluorescence micrographs of FITC-labeled *P. haemolytica* A2 with ethidium bromide (EB) added (magnification x 240)**

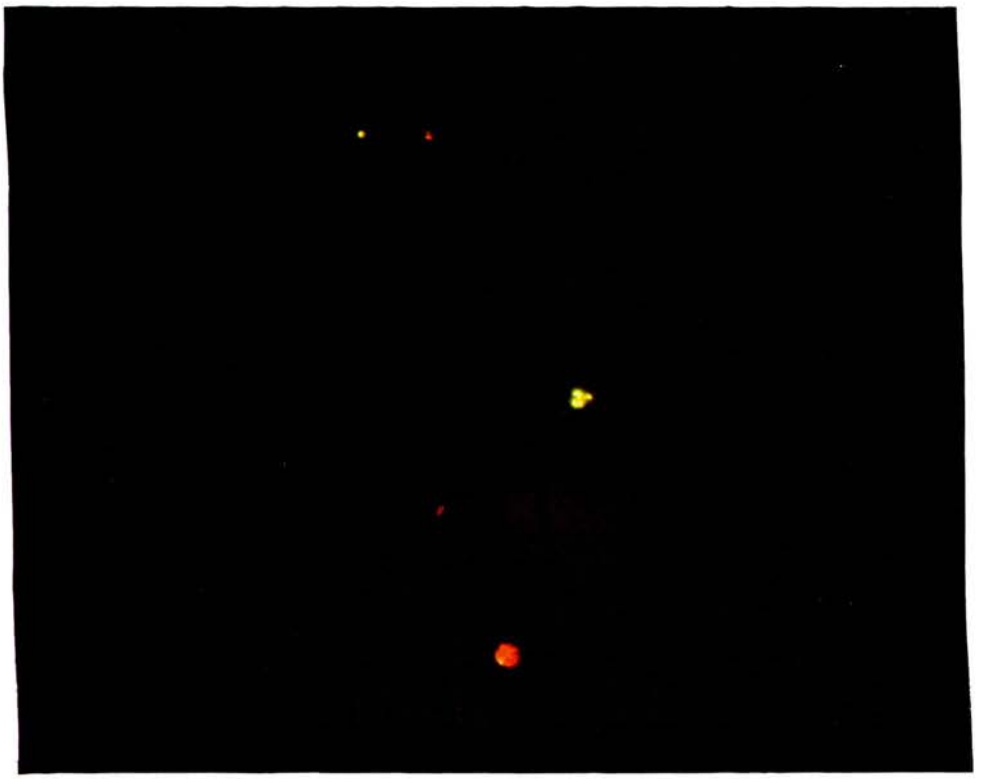

Plate 9.3. **Fluorescence micrograph of macrophages and phagocytosed FITC-labelled *P. haemolytica* A2 with ethidium bromide (EB) added. Intracellular bacteria remain green while extracellular bacteria fluoresce orange(magnification x 240)**

extracellular (orange) and intracellular (green) FITC-labeled *P. haemolytica* A2.

The results of the phagocytic activity of sheep alveolar macrophages were in agreement with that obtained with the mice experiment (Table 9.2). The mean number of sheep alveolar macrophages that showed phagocytosis of opsonised bacteria was significantly higher when the antisera used for opsonisation was taken from purified OMP-PS immunised animals ($p<0.05$). Phagocytosis of opsonised bacteria was poor when the antisera used for opsonisation was taken from crude OMP-PS or sham-vaccinated animals.

9.4 Discussion

The study using antibody-mediated phagocytosis assay was conducted to determine the level of opsonising antibodies to OMP-PS of *P. haemolytica* A2 present in the sera of mice and sheep vaccinated with the purified and crude preparation of the OMP-PS complex. The study has demonstrated that *P. haemolytica* A2 are readily labeled by FITC and when exposed to ethidium bromide the green to orange colour shift occurs.

Ultra violet-killed FITC-labeled *P. haemolytica* A2 was used in this assay thus avoiding the alteration of bacterial surface proteins important in phagocytosis. However, Drevet & Campbell (1991) showed that there was no difference between FITC-labeled live versus heat-killed *Listeria monocytogenes* with respect to the

percentage of macrophage associated bacteria which were internalised.

Sera from vaccinated sheep or mice which received purified OMP-PS antigen had high specific serum antibody titres to *P. haemolytica* A2 and significant increased opsonising activity than those from animals which were a) unvaccinated or b) vaccinated with crude complex.

The correlation of high percentage of opsonophagocytic activity and high IHA titres in the sera conferring protection in mice against *P. haemolytica* A2 challenge suggest that capsular antibody is important. The study also indicated that the sera from mice vaccinated with meningococcal group B vaccine poorly opsonise *P. haemolytica* A2 (results not shown). This in in agreement with the findings of Donachie *et al.*, (1991, unpublished data) who reported that the sera from sheep vaccinated with meningococcal group B vaccine do not opsonise *P. haemolytica* A2. The author suggest that the mechanism of phagocytosis might be species specific *in vitro.*

Macrophage phagocytosis assay using fluorescence microscopy has proved useful as an experimental tool for evaluating serum opsonic capacity and phagocytic cell function. The assay allows discrimination of bacteria which are bound to the external surface of a cell from those internalised by it. Measurement of internalised and externalised cell by fluorescence microscopy allows the asessment of the intracellular and opsonophagocytic functions of macrophages *in vitro*. The method

was used for studying phagocytosis of fluorescein-labeled *Listeria monocytogenes* by inflammatory mouse peritoneal macrophages (Drevets & Campbell, 1991). The results have indicated that this macrophage phagocytosis assay can be applied to *P. haemolytica.* This assay is simple, sensitive and reproducible and can be used to obtain information about phagocytic cell function or serum opsonic antibody content.

CHAPTER 10.0 GENERAL DISCUSSION

The aims of this study were to identify the prevalence and distribution of *Pasteurella haemolytica* serotypes causing pneumonic pasteurellosis in sheep and goats in Malaysia, assess the efficacy of a novel *P. haemolytica* iron-regulated protein (IRPs) vaccine in field trials and to isolate, characterise and assess the immunological significance of the polysaccharide capsule of *P. haemolytica* A2.

This general discussion examines the extent to which the original aims of this study have been realised, the questions which remain to be answered, and makes suggestions about the type of the research which may in the future provide additional useful information.

Pneumonic pasteurellosis remains a common and serious problem to the sheep and goats industry in many livestock producing countries including Malaysia. In the UK, pasteurellosis caused by *P. haemolytica* is the most important respiratory disease and is one of the most common bacterial infections of sheep (Gilmour and Gilmour, 1989). It has been estimated that not a single farm in Malaysia is free from the problem. The incidence of the disease is increasing and there is continuing need for vaccines to be used

in its control. However, the vaccines that were available to farmers are of questionable value and since protection of sheep by the *Pasteurella* vaccines are serotype-specific (Gilmour *et al.*,1983) there is a possibility that differences which may be present between the imported vaccine strains from the Malaysian field strains result in this poor efficacy. Therefore this thesis started with an epidemiological study to identify the serotypes of *P. haemolytica* involved in disease and determine their relative prevalence in the country. The dominant field strains isolated were compared with the UK strains for protein and LPS profiles in SDS-PAGE.

The serotyping study indicated that there was little difference in the relative frequency of occurence of A serotypes in the UK and in Malaysia. The serotypes isolated largely agree with the recognised pattern in sheep in the UK (Fraser *et al.*, 1982), Serotype A2 was by far the most common and the other A serotypes did not differ significantly in order of prevalence. However T biotypes appear to be rare in Malaysian isolates possibly because they are associated with temperate latitudes (Gilmour & Gilmour., 1989). The serotype distribution for the A serotypes in the present study is also similar to that found in a survey of ovine pasteurellosis cases at MRI during 1982-1993 (Quirie, 1994, unpublished data) and also in Northern Ireland (Ball *et al.*, 1993).

When electrophoretic protein and lipopopolysaccharide profiles of some common strains from both countries were compared by SDS-PAGE analysis there appeared to be no significant differences among the strains. Four to five major protein bands with

about twenty minor bands were shown to be present. The lipopolysaccharides profiles were of rough-type and designated type 3 (Davies et al., 1992). This in agreement with the earlier work by reported by Adlam *et al.*, (1989). However, it is in disagreement with the findings of Davies *et al.*, 1992 who showed that some isolates of A serotypes has smooth-type LPS. These workers used a different method of LPS extraction and different gel concentrations.

The epidemiological results may be of significance and the information can be used to design relevant control programmes involving management and disease prevention methods against ovine and caprine pasteurellosis in Malaysia as they give an indication of the serotypes most commonly found in pneumonic cases in the field. The study has indicated that British strains can be used in the vaccines for control of pasteurellosis in Malaysian farms.

Vaccines containing cell-free extract of of *P. haemolytica* serotypes A1, A6 and A9 have been shown to protect SPF lambs against experimental pasteurellosis caused by these serotypes (Gilmour *et al.*, 1983). The results of similar vaccine experiments with the A2 serotype were not succesful. Although some protection was observed this was never greater than 50% or often less (Gilmour *et al.*, 1983). An effective vaccine against A2, the most common serotype associated with sheep disease throughout the world is required.

New vaccines incorporating iron-regulated proteins (IRPs) have been developed. Molecular analyses of *P. haemolytica* cells, recovered from infected lambs, revealed the presence of these novel surface antigens expressed *in vivo* growth in the host. They were shown to be IRPs which are inducible in iron-deficient laboratory media (Donachie & Gilmour, 1988). Incorporation of IRPs antigens into SSE vaccines for *P. haemolytica A2* conferred significant protection against A2 challenge confirming their importance in immunity (Gilmour *et al.*, 1991). Subsequently, the efficacy of these novel vaccines containing 5As and 4Ts of *Pasteurella haemolytica* strains grown under restricted iron has been studied extensively in the SPF sheep models. Under field conditions, not many studies have been conducted. An extensive study using this novel vaccines were conducted in 2 large and 2 small farms in Malaysia involving 1000 animals. In this study the magnitude of antibody response against 35 kDa IRP and anti-A1 capsule measured by ELISA and IHA in animals immunised with Ovipast^{R}-9-IRP vaccine were examined.

The IRP vaccine elicited serum antibody responses against all the antigens tested confirming the immunogenicity of the vaccine components. Good antibody responses against the 35 kDa IRP antigens were associated with pure iron-regulated OMP components incorporated in the vaccines. Antibody response to 35 kDa IRP similar to this but using SPF lambs have been demonstrated by other investigators (Gilmour *et al.*, 1991). An ELISA to measure antibodies to a 35kDa IRP protein was used in most immunisation studies with IRP vaccine in SPF sheep models. This protein is used as general marker antigen for IRPs as it is assumed that all IRPs are produced in concert (Donachie, personal communication). However, it is clear from report that it is not

specific for *P. haemolyica* and in many situations the sera from sheep from areas thought to be free from *P. haemolytica* infection show high ELISA readings.

Capsular polysaccharide is found in large quantitities in the surface of bacteria and considered an important virulence factor of *P. haemolytica*, perhaps explaining the predominance of serotypes A2 and A1 in disease. Therefore high levels of IHA titres were demonstrated in the vaccinated and control animals. Antibody response to anti-A1 capsule is poor and this could be explained by the fact that our assay used an antigen capture system using monoclonal antibodies to A1 capsule, making it more specific.

The results so far obtained with the efficacy study of the new IRPs vaccines were encouraging and are in agreements with that of Donachie *et al.* (1984a) who demonstrated that high IHA and ELISA titres of antibodies to serotype antigens of A2 were obtained after infection with live A2. In SPF lambs the highest antibody responses also occur after challenge with live organisms (personal observation).

This field study investigated the immunologic responses to the these various antigens but no correlation to protection was investigated. As it is not possible to produce pneumonic pasteurellosis regularly in conventionally reared lambs by exposure to aerosols of *P. haemolytica*, the assessment of the effect of vaccination was based on the measurement of the serum antibody titres. A high degree of correlation between IHA antibody responses and protection has been demonstrated (Gilmour *et al.*, 1980).

Gilmour *et al.* (1983) found that the IHA titre of serum from sheep immunised with *P. haemolytica* SSE correlated on a group basis but not an individual basis with protection. The results of the same correlation between high anti-A1 capsular titres and protection have also been implicated in other studies (Gilmour *et al.*, 1979; Confer *et al.*, 1989). Similar data for usefulless of 35 kDa IRPs in the induction of protective immunity in pneumonia of sheep, have shown positive correlation between antibody titres and protection against disease (Gilmour *et al.*, 1991). The exact mechanisms by which antibodies against IRPs effect protection is at present unknown, but they may prevent iron uptake by the bacterium by blocking siderophore or transferrin binding, thereby reducing its ability to replicate quickly enough to cause disease.

Under field condition, a good antibody response could be achieved in animals immunised with the vaccines. However, multiple doses are required to achieve an optimal level of efficacy with this vaccine. When serological monitoring was extended to 4 months it was found that the serum antibody titres were almost the same for all the groups of vaccinated and control lambs. However group numbers were too small for definitive assessment.

To prevent loss from clinical illness, vaccination against pasteurellosis on an individual herd level is widely applied in enzootically infected regions in Malaysia. However until now, vaccination with traditional vaccines has not been able to cause a major reduction in the spread of the disease. The efficacy of the vaccination with a new IRP vaccine was evaluated by monitoring for antibodies against 35 kDa IRP, anti-A1

capsule and IHA antibody. In the trial region this vaccination greatly reduced the incidence of the disease.

In Malaysia, in most flocks that were vaccinated to prevent clinical disease, the lambs were vaccinated only once. Unless the animals were given booster vaccination, the titres of the maternally derived antibodies were low. One vaccination was far less effective in reducing *P. haemolytica* infection against natural challenge than two vaccinations (data not shown).

This study showed that using Ovipast-9-IRP^{R} vaccine and monitoring for relevant specific antibodies the effect of vaccination on the incidence of pasteurellosis in a population can be seen.

Another candidate for a future *Pasteurella* vaccine is the capsular polysaccharide antigen of *P. haemolytica* A2. The capsule is a linear heteropolymer of α-(2→8)-linked N-acetylneuraminic acid. The capsule is a virulence factor of *P. haemolytica* A2 and similar to other capsulated pathogens, serves to protect the organism by antiphagocytic mechanism. An outer membrane protein-polysaccharide (OMP-PS) complex was succesfully prepared from an ovine isolate of *P. haemolytica* serotype A2 by precipitation from log phase culture supernatant and subsequent purification by column chromatography as previously described by Moreno *et al.*, 1985 in preparing OMP complexed to group B polysaccharide of *N. meningitidis*. The results have shown that the optimum production of the complex was determined to be in 6 hour culture and

comprised of protein and polysaccharide (4:1 w/w) and low in lipopolysaccharide content. This is in agreement with earlier work reported by Moreno *et al.* (1985) who showed that the OMP complexed to group B polysaccharide of *N. meningitidis* revealed low levels of nucleic acid and LPS content and the complex could be obtained optimally in 6 hour culture.

The importance of a purified OMP-PS complex as a protective antigen was demonstrated in mice and sheep. The complex was immunogenic in mice and adult sheep with induction of humoral anti-capsular antibody as measured in the indirect haemagglutination (IHA) test and with OMP in immunoblotting. Direct immunisation of mice with the complex vaccine demonstrated significant protection against A2 infection. Induction of anti-capsular antibody and correlation with protection were also demonstrated by other investigators (Gilmour *et al.*, 1979; Confer *et al.*, 1989). However, Gilmour *et al.* (1983) reported that although cell extract vaccines of some serotypes of *P. haemolytica* were protective in SPF lambs, inactivated A2 antigens were relatively ineffective. The low immunogenicity of the capsule of A2 serotype is thought to be due to its chemical structure, an α-(2→8)-linked polymer of N-acetylneuraminic acid which was identical to the colominic acid polymers of *N. meningitidis* group B. This colominic acid has a very low immunogenicity in rabbilts and man, and is thought to be due to identical chemical structures on certain gangliosides on the surface of host cells (Glode *et al.*, 1977; Kasper *et al.*, 1973; Lifely *et al.*, 1987), loose configuration and sensitivity to host neuraminidases (Lifely *at al.*, 1987) may all contrbute to decrease immunogenicity. The bacterial polymer is thus not identified as foreign to the host,

permitting evasion of its immune system. As a consequence, the capsular polysaccharide does not induce specific antibodies. However, the capsular polysaccharide noncovalently complexed with outer membrane protein has shown promising results when tested in mice and adult sheep.

The sera from two-year old sheep immunised with OMP-PS passively protected mice against A2 challenge. This is in agreement with earlier work reported by Moreno *et al.*, 1983 who demonstrated that monoclonal antibodies against meningococcal polysaccharide group B protected mice passively against challenge with live organisms. Three-month old lambs immunised with the same vaccine did not respond serologically and this sera did not passively protect mice. Adlam *et al.* (1987) also reported that the age of animals at time of vaccination is important as he demonstrated the non-responsiveness of young lambs to purified antigens of *P. haemolytica.* The poor antibody response in the young lambs are likely to be due to immune tolerance. In human, the polysialidated proteins that are found in the embryonal and newborn brain tissue are identical to the repeating units of sialic acid in *N. meningitidis* group B polysaccharide (Finne *et al.*, 1982, 1983). The situation is possibly the same with the young lambs, and consequently, the animal being tolerant to this self epitope results in poor antibody response to purified OMP-PS of *P. haemolyica A2* complex vaccine.

In vitro studies using ovine and murine macrophages indicated that the mechanism of protection is antibody mediated phagocytosis as the opsonophagocytic activity of immune sera could be demonstrated. This is in agreement with the work

reported by Brodgen *et al.* (1989) who suggested that correlation of anti-capsular antibody and protection may operate by inhibiting adhesion of bacteria to the mucosal surface as well as augmenting phagocytosis. The correlation of high opsonic activity and high IHA titres in the sera with protection of mice against *P. haemolytica* A2 challenge suggest that capsular antibody is important. It has been reported that sera from sheep vaccinated with meningococcal group B vaccine do not opsonise *P. haemolytica* (Donachie, 1991 unpublised data). The author suggested that the phagocytic mechanism might be species specific.

Previous work on *P. haemolytica* showed that serotype A2 antigens, heat killed cells together with cellular extracts produced by treatment with sodium salicylate were of reduced immunogenicity compared to similar antigens from other A serotypes. The results presented here demonstrate that solid protection against serotype A2 is produced in mice receiving sheep sera containing antibody against purified OMP-PS complex of *P. haemolytica* A2 and its potential to immunise adult sheep and suggests that passive protection of lambs against A2 infection is obtainable.

The protective property of the *P. haemolytica* A2 OMP-PS complex suggests that it should be considered as a possible vaccine component for sheep vaccines although the preparation procedure might be too elaborate for commercial firms.

The traditional approach to vaccine formulation has been to incorporate all of the most commonly isolated serotypes but this leads to complex formulations which

imposes a very heavy antigenic load on vaccinated animals. The identification of the shared protective antigens is therefore important in the development and improvement of this type of vaccine as their inclusion will reduce the overall quantity of the antigen required.

With regard to future research, a detailed understanding of the exact pathway by which *P. haemolytica* obtain iron from transferrin is necessary to allow the production of even more effective vaccines based on IRPs. In addition, because natural polysaccharides production is low, some means of improving its yield has to be found if it is to be an economic component of vaccines. On the evidence of these results, it would seem that new *Pasteurella* vaccine should contains IRPs, capsule, leukotoxin and perhaps LPS if they to confer a very high measure of immunity.

Inactivated whole cell *Pasteurella* vaccines will continue to be the major type of vaccine available but with the rapid discovery and purification of important immunogens, through the use of recombinant DNA techniques in particular, antigenically well defined vaccines should start to emerge. When these are combined with the greater knowledge of the host's immune response and the immunopotentiating power of the new adjuvants the "second generation" of super vaccines will have arrived. However in the harsh world of veterinary and agricultural economics these vaccines will have to be affordable and inevitably compromises will be encountered on the way to their final application.

To conclude, the major points arising from this work are: there was little difference in the relative frequency of occurence and electrophoretic protein and LPS profiles of A serotypes in the UK and Malaysia; the new *P. haemolytica* IRP vaccine generated an immune responses as measured in ELISA and IHA tests and appeared to correlate with protection against pasteurellosis; and the potential af a *P. haemolytica* A2 OMP-PS complex vaccine to immunise adult sheep was demonstrated and suggests that passive protection of lambs against A2 infection is obtainable.

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PUBLICATIONS ARISING FROM THE THESIS

1. MOHAMAD, M., QUIRIE, M. & DONACHIE, W. (1993).
   Prevalence of *Pasteurella haemolytica* serotypes isolated from ovine and caprine pasteurellosis in Malaysia.
   In Proc. 16th. Malaysian Society of Animal Production (MSAP). Langkawi, 7-10 July, 1993.

2. MOHAMAD, M. & DONACHIE, W. (1993)
   Protection of mice against *Pasteurella haemolytica* A2 challenge using a protein-polysaccharide complex vaccine.
   In Proc. of the 5th. Veterinary Association of Malaysia (VAM) Congress. Air Keroh, 7-10 October, 1993.

PROTECTION OF MICE AGAINST *PASTEURELLA HAEMOLYTICA* A2 CHALLENGE USING A PROTEIN/CAPSULAR POLYSACCHARIDE COMPLEX VACCINE

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SUMMARY

An outer membrane protein-capsular polysaccharide (OMP-PS) complex was prepared from an ovine isolate of *Pasteurella haemolytica* serotype A2 by precipitation from log phase culture supernatant and subsequent purification by column chromatography. This complex comprised protein and polysaccharide (4:1 w/w) with traces of lipopolysaccharide. The purified OMP-PS vaccine stimulated an antibody response to capsule in the indirect haemagglutination (IHA) test and to OMP in immunoblotting. In contrast to crude OMP-PS and whole bacteria vaccines the purified complex protected mice against challenge with *P. haemolytica* A2.

INTRODUCTION

Pneumonia associated with *Pasteurella haemolytica* is a major cause of economic loss to the sheep and goat industry in Malaysia. Protection against the A2 serotypes, which is the predominant ovine isolate from field cases, has proven difficult to demonstrate with cell extract vaccines. Serotype A2 has a capsular structure; α(2→8)-linked N-acetylneuraminic acid identical to the colominic acid of *Neisseria meningitidis* group B and *Escherichia coli* K1 and is known to be poorly immunogenic (1). Inoculation with monoclonal antibody against *N. meningitidis* group B capsule and outer membrane protein-capsular polysaccharide (OMP-PS) complex meningococcal vaccine protected mice against challenge with *N. meningitidis* group B and *P. haemolytica* A2 strains (3,6). The result prompted research to formulate *P. haemolytica* vaccine with a similar composition to meningococcal vaccine to evaluate its efficacy against experimental pasteurellosis.

MATERIALS AND METHODS

Bacteria and growth condition: *Pasteurella haemolytica* A2 (strain EO 200) was grown in nutrient broth (Gibco No.2) for 18 h at 37°C. Large quantities of bacterial cells for capsule extraction were produced by inoculating 5 litres nutrient broth with 50 ml seed broth and incubating at 37°C for 6 h on orbital shaker.

Indirect haemagglutination (IHA) test: The rapid method described by Fraser *et al.* (5) was used.

Mouse protection model: C57 black mice (6-8 wk old) were used for immunisation and protection (4).

Preparation of the OMP-PS complex vaccine: Preparation of crude and purified complex was based on the method used by Moreno *et al.* (6) for purifying OMP-PS complex from *N. meningitidis* group B.

Heat-killed organisms and extract: This preparation was based on the procedure used by Donachie (2).

Meningococcal group B vaccine: This was kindly donated by Dr. R. Lifely, Wellcome Biotech. Kent, UK. The typical composition of vaccine complex is 48.6% sialic acid, 46.8% protein and 4.1% lipopolysaccharide (LPS).

Immunoblotting: Whole cells preparations of *P. haemolytica* were separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on nitrocellulose paper (Sartorius). The blots were probed with mouse antisera and sheep anti-mouse IgG. HRP conjugate was used to detect bound mouse antibodies.

RESULTS

The composition and yield of the OMP-PS complex preparation are shown in Table 1. Assay of the serum antibodies of mice immunised by the purified OMP-PS in the IHA test indicated high titres to A2 in a number of mice. The titres range from $^1/_{128}$ to $^1/_{256}$.

Significant protection ($P<0.005$) was conferred on mice immunised with the purified OMP-PS complex and meningococcal group B vaccine (Table 2). Sera from vaccinated and control mice were examined in immunoblots against *P. haemolytica* A2 cells to determine the antibody response to cellular proteins. A very strong response was observed at around 42 kDa and 26 kDa.

Table 1. Protein and sialic acid determined in a preparation of OMP-PS complex of *P. haemolytica* serotype A2

Preparation	Protein (mg/ml)	Sialic acid (mg/ml)	Ratio of protein/sialic acid
Crude OMP-PS complex	1.1	0.72	1.5
Purified OMP-PS complex	0.08	0.02	4.0

Table 2. Counts[a] of *P. haemolytica* A2 in the livers of unvaccinated control mice and mice vaccinated with *P. haemolytica* A2 antigens after challenge[b].

Group	Vaccine	Vaccine Dose	Mean count in liver after 6 h (cfu)[a]	p*
1	Heat killed organisms	2.9×10^8 cfu/ml	4.499	NS
2	Heated culture extract	2.9×10^8 cfu/ml	4.298	NS
3	Crude OMP-PS complex	10 µg sialic	4.147	NS
4	Purified OMP-PS complex	10 µg sialic	1.253	<0.002
5	Meningococcal gp.B vaccine	1 µg	1.803	<0.005
6	Unvaccinated control		5.494	

a - Counts = colony forming units (cfu) expressed as log values
b - Challenge dose (1.02×10^7 cfu)
* - When compared with the control group in the Mann-Whitney Ranking Test

DISCUSSION

Significant protection (P<0.005) against *P. haemolytica* A2 infection was conferred on mice by a vaccine containing purified OMP-PS complex. In contrast, the crude complex and the heat-killed whole bacteria vaccines did not give significant protection. A similar level of protection against A2 challenge has been demonstrated previously in mice injected with meningococcal group B vaccine (3). The purified OMP-PS complex stimulated an antibody response to capsule in the IHA test and to OMP in immunoblotting. Immunoblotting of this sera demonstrated strong responses to OMP with apparent molecular weights of 42 kDa and 26 kDa. This experiment has shown that purified OMP-PS can act as a very effective vaccine in mice against challenge with A2. Further studies are now in progress to establish the immunogenicity of this complex in sheep.

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PREVALENCE OF *Pasteurella haemolytica* SEROTYPES ISOLATED FROM OVINE AND CAPRINE PASTEURELLOSIS IN MALAYSIA

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Pasteurellosis due to infection with *Pasteurella haemolytica* or *P. multocida* are major diseases of ruminants of widespread geographical importance. The disease causes serious economic loss to many major livestock producing countries. Prevention of pasteurellosis has proven difficult. While some efforts have been directed towards minimising exposure to some factors associated with the disease, vaccination is accepted as the most effective and practical method of control.

To make a vaccine specifically intended for sheep and goats in Malaysia it is important to establish the prevalence of diferrent serotypes in the country. The purpose of the present study was to examine the serotype strains of ovine and caprine origin submitted for serotyping at Moredun Research Institute, Edinburgh, UK and to determine the range of serotypes for the year 1989-1991.

The strains submitted had been isolated at MARDI, Universiti Pertanian Malaysia and Veterinary Research Institute from sheep and goats throughout Malaysia which were either clinically abnormal or had pathological condition at postmortem. Strains were received on blood agar plates or slopes or freeze-dried and were serotyped either directly from the original culture or after subculture on 5% sheep blood agar. Strains were serotyped by a rapid indirect haemagglutination method as described by (2).

A total of 215 strains were submitted for serotyping (Table 1). The predominant serotypes were A2, A9, A7 and A1 (38.5%, 16%, 14% and 13% respectively). Three biotype A serotypes (A5, A6 and A11) were isolated in smaller number. The biotype T was very rare (only 2%). Fourteen percent strains were not typable by the indirect haemagglutination method.

P. haemolytica biotype A serotype 2 has been found to be the most common serotype isolated from sheep and goat pneumonic pasteurellosis in Malaysia.The serotype A2 was also the most prevalent serotype recovered from sheep farms in the United Kingdom, (5) and New Zealand (6). Of the 215 isolates of *P. haemolytica*, 32 (15%) were untypable . This is typical of such surveys, e.g (1) and (3) found 12%, 28% and 34% respectively of their isolates were untypable.

The range of serotypes isolated from lungs suggests that all the common serotypes should be included in vaccines against pneumonic pasteurellosis since immunity is serotype specific (4). It is necessary to monitor continously the prevalence of the various serotypes and to investigate the pathogenicity of new seroptypes strains in order that the appropriate major serotypes can be incorporated in vaccines.

Acknowledgement. - We are indebted to the University Pertanian Malaysia and Veterinary Research Institute who referred strains.

Table 1. The prevalence of serotypes of *P. haemolytica* isolated from sheep and goats pasteurellosis in Malaysia.

Serotype	1989*	1990	1991	Average
A1	7	11	28	15
A2	14	36.5	55	35
A5	7	-	-	2
A6	7	-	-	2
A7	11	14	8	11
A9	36	7	-	14
A11	-	2	-	<1
T3	7	-	-	2
UT	11	27	9	15
Total Strains Typed	80	50	85	215

* - Expressed as a percentage of the total number of strains examined.

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APPENDIX 1

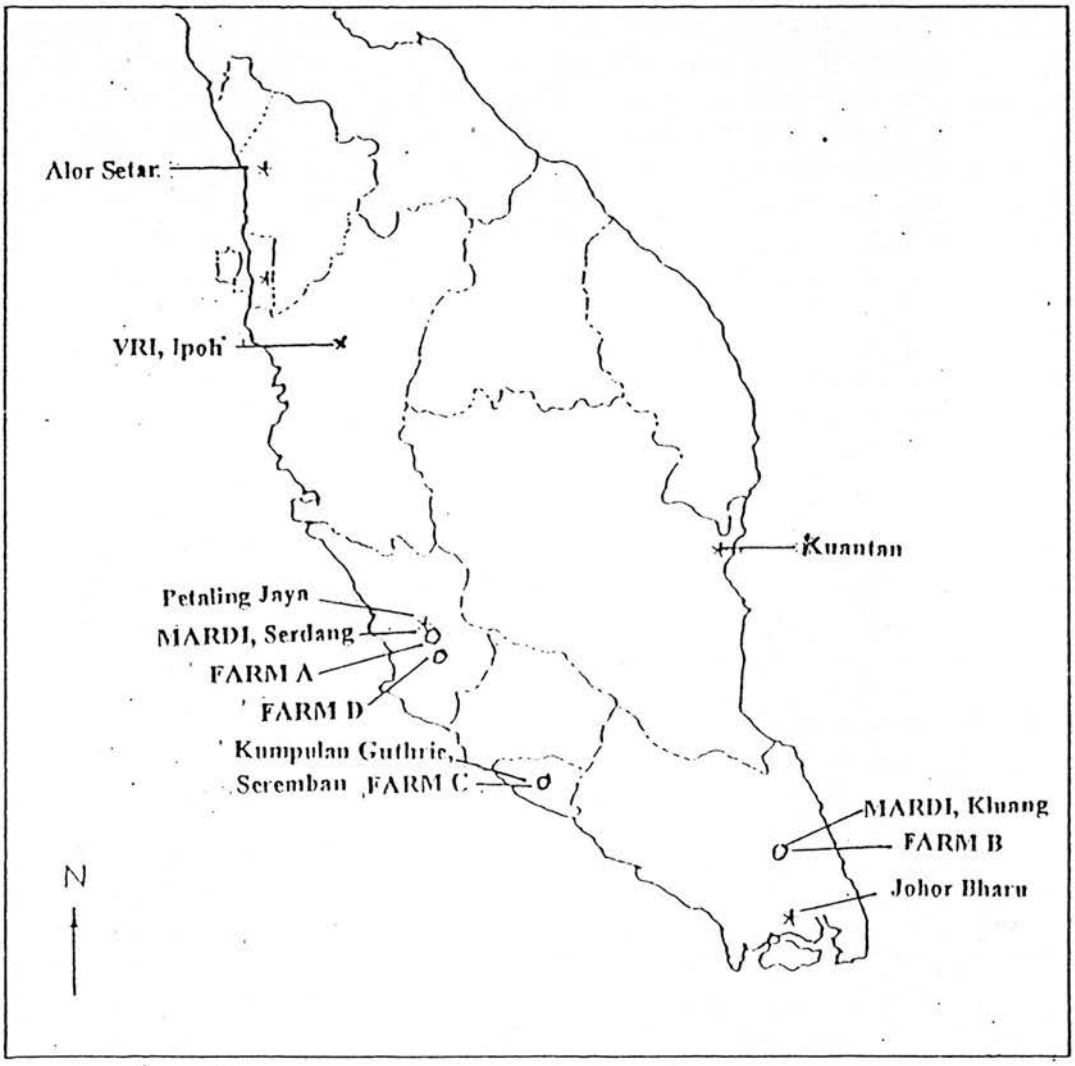

Fig. 5.0 Map showing locations of; a) Laboratories where *P. haemolytica* strains referred for serotyping b) Field vaccine trial farms.

APPENDIX II

Table A (Data from Chapter 4.1)

Clinical condition reported for sheep and goats suspected of pasteurellosis during 1989-1993.

Animal	Clinical condition				
	Death	Respiratory signs	Weak/unthrifty	Other*	Total
Sheep -Adults	60	15	10	30	115
-Lambs	196	58	42	15	311
Goats -Adults	12	17	12	15	56
-Kids	83	28	18	10	139
Totals	351	118	82	70	621

* - mastitis (in ewes and does) and perinatal mortality

Table B (Data from Chapter 4.1)

Principal pathological abnormality observed in sheep and goats yielding *Pasteurella haemolytica* strains during 1989-1993.

Animal	Pathological condition				
	Pneumonia	Upper Respiratory Tract condition	Septicaemia	Other*	Total
Sheep -Adults	94	44	10	18	166
- Lambs	22	0	132	0	154
Goats -Adults	17	9	6	13	45
-Kids	14	0	58	0	72
Totals	147	53	206	31	437

* - mastitis and enteritis

Table C (Data from Chapter 4.1)

Organ distribution of *Pasteurella haemolytica* strains examined during 1989-1993.

SAMPLES	SHEEP				GOATS				TOTALS
	Pneumonic lungs	Nasal swabs	Lymph nodes*	Others**	Pneumonic lungs	Nasal swabs	Lymph nodes*	Others**	
No. of samples	256	90	40	55	95	35	32	18	621
P. haemolytica	256	18	24	22	95	7	9	6	437
Mixed infections***	17	8	8	42	8	4	13	8	108
Total No. of isolates	273	26	32	64	103	11	22	14	545

* - Retropharyngeal and submandibular lymph nodes only were examined.

** - Spleen, liver or kidney

***- *Pasteurella multocida, Haemophilus somnus, Actinobacillus* spp., *Salmonella* spp., *Escherichia coli* and *Listeria monocytogenes*

Table D (Data from Chapter 5.0)

Mean IHA antibody response to *P. haemolytica* antigens in sheep vaccinated with Ovipast-9-IRP vaccine.

FARM A

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.395	0.415
1	0.85	0.34
3	0.5573	0.47
5	1.415	0.36

FARM B

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.385	0.35
1	1.225	0.385
3	0.7968	0.275
5	1.63	0.335

FARM C

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.4068	0.3167
1	1.371	0.378
3	1.015	0.4
5	1.708	0.4808

FARM D

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.45	0.4568
1	0.85	0.442
3	1.667	0.6247
5	1.84	0.7247

Table E (Data from Chapter 5.0)

Antibody reponse to *P. haemolytica* A2 35 KDa IRP in sheep vaccinated with Ovipast-9-IRP vaccine

FARM A

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.2218	0.2443
1	0.7968	0.1755
3	0.5146	0.1652
5	1.032	0.314

FARM B

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.3293	0.1624
1	1.172	0.2078
3	1.085	0.2408
5	1.171	0.2984

FARM C

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.216	0.2913
1	1.295	0.3093
3	0.786	0.4985
5	1.399	0.5073

FARM D

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.2429	0.2319
1	1.332	0.2858
3	0.812	0.2742
5	1.375	0.2319

Table F (Data from Chapter 5.0)

Antibody response to *P. haemolytica* anti-A1 capsular antigen in sheep vaccinated with Ovipast-9-IRP vaccine

FARM A

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	6	5.8
1	56	9.4
3	16	10.8
5	144	9.6

FARM B

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	5	6
1	22	11
3	18	16
5	96	11

FARM C

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	5	10
1	22	11
3	18	18
5	48	11

FARM D

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	8	9.6
1	64	11.4
3	32.4	16
5	80	13.6

Table G (Data from Chapter 5.2)

Serum IHA titres (a), antibody response to 35 KDa IRP of *P. haemolytica* A2 (b)and A1 capsular antigens (c) in sheep vaccinated with Ovipast-9-IRP vaccine in challenge exposed experiment.

a

Time	Mean 1 HA Titres	
Week	IRP-vaccinated	Non-vaccinated
0	9.6	6.4
1	56	9.4
2	16.4	10.8
3	8	10.95
4	49	13.6
5	144	16.4
6	166.4	15.6

b

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.71	0.41
1	1.5	0.7
2	0.9	0.6
3	1	0.65
4	0.95	0.6
5	1.2	0.5
6	1.37	0.38

c

Time	Mean OD at 492 nm	
Week	IRP-vaccinated	Non-vaccinated
0	0.12	0.11
1	0.24	0.14
2	0.16	0.15
3	0.14	0.12
4	0.16	0.1
5	0.35	0.18
6	0.29	0.19

Table H (Data from Chapter 7.2)

Counts of *P. haemolytica* A2 in livers of control mice and mice immunised with monoclonal antibodies with high 1HA activity (mAb 34A10 and mAb 62A12)

Group	Liver counts
mAb 34A10	6.12
mAb 62A12	4.57
Control +ve	4.32
Control -ve	6.77

Table I (Data from Chapter 7.2)

Counts ($\log_{10}$)of *P. haemolytica* A2 in livers of control mice and mice immunised with meningococcal polysaccharide-IMP vaccine

Group	Liver counts
Vaccinates	4
Placebo	7.29
Control	7.09

Table J (Data from Chapter 8)

Serum IHA antibody titres of adult sheep vaccinated with crude and purified OMP-PS complex vaccines and the sham-vaccinated group.

Time	OD at 492 nm		
Week	Crude OMP	Purified OMP	Sham-vaccinated
0	9.5	0.5	8
1	9.5	1	4.5
2	9.5	12.5	2
3	10.5	13	2
4	24.5	20	1.5
5	24.5	52	1.5
6	40.5	96	5.5

Table K (Data from Chapter 8)

Serum antibody titres of the lambs vaccinated with crude and purified OMP-PS complex vaccines and sham-vaccinated group.

Time	OD at 492 nm		
Week	Crude OMP	Purified OMP	Sham-vaccinated
0	2	2	2
1	2.5	2.5	2
2	2	2	2
3	2.5	2.5	2
4	3	2.5	2
5	2.5	2.5	2
6	2.5	3	3